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Modeling developmental plasticity in variable environments through gene duplication: a case for phytochromes and gibberellin3-oxidases' role in temperature-dependent seed germination

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Abstract

Seed germination is a critical developmental transition in plants and is regulated by complex combinations of environmental cues that restrict its timing to occur over the widest possible range of conditions suitable for subsequent survival. To investigate the genetic mechanisms by which seeds achieve this restriction, we characterized the functional diversification of two duplicated gene families, phytochromes and gibberellin3-oxdiases, involved in temperature dependent seed germination. Germination responses were recorded of single and multiple PHY and GA3ox functional knockout mutants after exposure to various temperature treatments reflecting seasonal conditions. PHYE was the only copy necessary for germination in cold temperatures after a cold imbibition treatment, while PHYB was an important contributor to germination after seeds did not experience a dormancy-breaking imbibition treatment. GA3ox2 was the most important GA3ox contributor to germination in a range of temperature conditions while GA3ox1 and GA3ox3 appeared to contribute redundantly to germination with GA3ox2, the first evidence of GA3ox3's role during temperature-dependent germination. The diversification of these genes during germination suggests that gene duplication plays an important role in regulating a plant's response to complex seasonal environments. We then constructed a basic two-step genetic pathway model to investigate how environmental sensitive and/or functionally diversified duplicated genes are able to restrict a physiological response, such as germination, to combinations of environmental cues. Identical diversifications in duplicated upstream and downstream genes resulted in the most precise restriction of responses around optimal environmental conditions. Moreover, environmental sensitive diversification within duplicated genes was required in order to elicit a physiological response to more than one set of optimal environmental conditions supporting the importance of diversified duplicated genes in achieving developmental plasticity in variable seasonal environments.

1.1 Introduction

Seed germination is a critical developmental transition in plants as it determines the environment that plant will be exposed to for the rest of its life. As a result, the timing of seed germination has significant consequences on a plant's later life history, phenology, and overall lifetime fitness in a given environment (Simons and Johnston 2000; Finch-Savage et al. 2006). The timing of seed germination has even accounted for more than 70% of a plant's variation in fitness among genotypes in a study with the model plant *Arabidopsis thaliana* (Donohue, Dorn et al. 2005). Unlike humans, plants don't have the luxury of packing up their things and moving to a new area if they don't like the neighborhood. Once they germinate, plants must either adjust to their surrounding environment or perish – making the "decision" to germinate or not, truly a matter of life and death.

In order to germinate at the appropriate time of the year in environment conditions suitable for subsequent growth and survival, seeds must be able to sense and respond to complex combinations of environmental cues. These cues ultimately control germination through two interrelated processes: the induction / breaking of dormancy and the promotion of germination after dormancy has been broken (Baskin and Baskin 1998). Dormancy is defined as the failure of a viable seed to germinate under favorable environmental conditions that may promote germination later (Baskin and Baskin 2004). Once seeds have matured, they may either be dispersed in a non-dormant or dormant state (Baskin and Baskin 2004). In the latter case, the seed is considered to be in 'primary dormancy' which can be broken, for example, by a period of dry after-ripening (the time in an environment occurred by a dry seed after it is dispersed) or an exposure

to cold temperature conditions (Baskin and Baskin, 1986; Davis 1930). If the seed is non-dormant on the other hand, it can either germinate immediately, given the presence of permissible environmental conditions, or wait until these permissible conditions arrive. A non-dormant seed may also enter 'secondary dormancy', which requires a dormancy breaking environmental treatment, such as exposure to cold temperatures during imbibition, before it can germinate under favorable conditions (Allen and Meyer 1998; Simpson 2007).

Among the many environmental cues involved in this transition from dormancy (or lack thereof) to germination, temperature is of particular importance on both a molecular and ecological level (Derkx and Karssen 1993). On the molecular level, temperature influences expression multiple genes and/or protein activities involved in the seed germination pathway (Yamauchi, Ogawa et al. 2004; Heschel, Selby et al. 2007). For example, cold temperatures up-regulate abscisic acid (ABA) catabolism and gibberllic acid (GA) metabolism resulting in the breaking of dormancy and/or the promotion of seed germination (Hilhorst and Karssen 1992; Yamauchi, Ogawa et al. 2004). Warm temperatures, on the other hand, up-regulate ABA metabolism and GA catabolism preventing germination by either inducing or maintaining seed dormancy (Karssen, Brinkhorst-Van der Swan et al. 1983; Yamaguchi 2008).

On the ecological level, temperature varies both seasonally and geographically and a seed's germination response to these variable environments can affect that plant's life history characteristics and phenology (Donohue 2002). For example, winter-annual species germinate in response to a period of warm after-ripening followed by exposure to cold resulting in overwintering as a rosette and then flowering in the spring. (Baskin

and Baskin 1972; Baskin and BASKIN 1986). Seeds that experience a prolonged exposure to cold on the other hand (ie – during winter conditions) may germinate, flower and disperse their seeds all in single spring season (Baskin and Baskin 1998). Thus, germination responses to temperature cues reflecting seasonal conditions can alter subsequent life-history traits and select for phenologies (ie – the timing of these life-history traits) most suitable for survival in that given environment (Donohue 2005). Moreover, global temperatures are expected to shift as a result of climate change (Houghton 1992; Walther, Post et al. 2002). Thus, understanding the genetic basis of temperature-dependent seed germination is important not only for understanding how plants currently adapt to their heterogeneous environments but also for predicting how they might respond to various climate change scenarios in the future (Walther, Post et al. 2002; Kelly and Goulden 2008).

Gene duplication is one mechanism by which plants may respond to complex environmental conditions and adapt to variable seasonal environments (Smith-Gill 1983; Blanc and Wolfe 2004; Donohue 2005). This is achieved in multigene families that not only contribute to the same physiological process but that have copies with functionally diversified roles during this process in response to different environmental conditions. For example, one gene copy within a functionally diversified multigene family may regulate a plant's germination response to cold conditions while another copy may regulate its response to warm conditions. Only when both gene copies are present however can the plant regulate the same physiological process (e.g.- germination) under both sets environmental conditions. Thus, gene duplication is an important genetic mechanism by which plants can evolve precise developmental responses in variable

seasonal environments. In order to understand this importance within the context of temperature-dependent seed germination, we must first identify which gene copies (within a multigene family involved in the germination pathway) actually contribute to germination. Then we may characterize the functional diversification of these contributing gene copies in response to combinations of temperature conditions reflecting seasonal environmental cues.

Phytochromes are among the most important environmental sensors in plants, and they regulate a seed's response to light, as well temperature, during seed germination (Reed, Nagatani et al. 1994; Poppe and Schafer 1997; Shinomura 1997; Whitelam and Devlin 1997; Ritchie and Gilroy 1998; Donohue, Heschel et al. 2007; Heschel, Selby et al. 2007). In the model plant *A. thaliana,* multiple gene duplication events have resulted in five different genes (denoted PHYA through PHYE) that encode for five distinct phytochromes (Sharrock and Quail 1989; Clack, Mathews et al. 1994; Mathews and Sharrock 1997). These phytochromes are photoreversible biliproteins that are synthesized in their inactive red-absorbing form (Pr) and converted by red light to their bioactive far-red absorbing (Pfr) which mediates several plant processes (including germination) in response to light (Borthwick, Hendricks et al. 1952; Casal and Sánchez 1998; Franklin and Whitelam 2004). PHYA and PHYB are the most important regulators of germination in response to light, with PHYA being important under far-red light and PHYB important under red-light (Shinomura, Nagatani et al. 1994; Poppe and Schafer 1997)

Recently however it has been shown that different duplicated phytochrome genes vary in their contribution to germination in response to temperature as well (Donohue,

Heschel et al. 2007; Heschel, Selby et al. 2007). For example, PHYA plays an important role in promoting germinating at warmer temperatures while PHYE plays an important role at colder temperatures (Heschel, Selby et al. 2007). PHYB has been shown to be an important contributor to germination across a range of temperature conditions, particularly if seeds don't experience a cold dormancy-breaking stratification (imbibition in the dark) treatment, while PHYD's role in promoting germination is most pronounced after seeds are exposed to warm, dormancy-inducing imbibition (dark) temperatures (Heschel, Butler et al. 2008).

Active phytochromes promote seed germination by up-regulating the production of bioactive GAs, which promote germination by degrading the seed coat and stimulating embryonic growth (Karssen, Brinkhorst-Van der Swan et al. 1983; Hilhorst and Karssen 1992; Koornneef and Karssen 1994; Ogawa, Hanada et al. 2003; Ritchie and Gilroy 1998; Yamauchi, Ogawa et al. 2004). Specifically, phytochromes up-regulate the expression of gibberellin3-oxidases, a class of 2-oxoglutarate-dependent dioxygenases that catalyze a final step in the production of bioactive GAs (Yamaguchi, Smith et al. 1998; Mitchum, Yamaguchi et al. 2006; Yamaguchi 2008). Bioactive phytochromes stimulate this downstream up-regulation by inhibiting the PIL5 protein which inhibits GA production and also promotes ABA production (Oh, Yamaguchi et al. 2006; Seo, Nambara et al. 2009).

These gibberellin3-oxidases (GA3oxs) are another example of a duplicated gene family with copies that have diversified in their sensitivity to temperature during seed germination and other developmental plant processes (Lester, Ross et al. 1997; Xu, Gage et al. 1997; Mitchum, Yamaguchi et al. 2006; Yamaguchi 2008) In *A. thaliana* there are

four GA3ox gene copies (GA3ox1 through GA3ox4) (Chiang, Hwang et al. 1995; Williams, Phillips et al. 1998; Yamaguchi, Smith et al. 1998). GA3ox1 has been shown to be up-regulated by cold temperature conditions and is subject to feedback regulation by GA whereas GA3ox2 is not (Yamauchi, Ogawa et al. 2004; Mitchum, Yamaguchi et al. 2006). Unlike phytochromes however, much less is known about the functional diversification of these GA3ox copies (in particular GA3ox3 and GA3ox4) in regulating a seed's germination response to dormancy-inducing and dormancy-breaking temperature conditions that reflect seasonal environments.

The purpose of these first sets of experiments therefore is to characterize the functional diversification of duplicated phytochromes and gibberellin3-oxidases during temperature-dependent seed germination in order to investigate the genetic basis by which seeds respond to combinations of environmental cues in variable environments. This leaves us with two central questions to be answered: 1) which duplicated PHY and GA3ox genes contribute to germination in response to combinations of temperature conditions? And 2) how are these copies functionally diversified with respect to temperature-dependent germination? To answer these questions, combinations of single and multiple PHY and GA3ox functional knock-out mutants in the model plant *Arabidopsis thaliana* were exposed to combinations of dormancy-inducing and dormancy-breaking temperature treatments that reflect different seasonal environments. The germination responses were recorded of fresh and dry-after ripened seeds after a period of dark, wet imbibition (or 'stratification treatment') at various temperatures before they were transferred to white light and allowed to germinate at various temperatures. We found both PHY and GA3ox copies displayed varying degrees of

functional diversification during temperature-dependent seed germination suggesting the importance of gene duplication in achieving developmental plasticity in response to complex seasonal environments.

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1.2 Methods

1.2.1 Diversification of PHY & GA3ox Copies in response to dormancy-inducing/breaking conditions

To investigate the functional diversification of PHY and GA3ox copies in regulating temperature-dependent seed germination, we compared germination responses of various PHY and GA3ox mutants to their background ecotype (either Landberg erecta (Ler) or Colombia (Col)) following various combinations of dormancy-inducing and dormancy-breaking stratification treatments (imbibition of seeds in the dark). Background ecotypes of mature, fresh seeds are expected to be in a non-dormant state when harvested. They may be induced into secondary dormancy by warm temperatures during imbibition in the dark (i.e. – a warm stratification treatment). Mutants that significantly reduce germination under a given combination of temperature treatments provides support for that gene copy's role in contributing to germination under those temperature conditions. Conversely, if a loss-of-function mutant does not break dormancy (i.e. – not germinate) in conditions that break dormancy (i.e.-germinate) in the wild-type, this suggests that the knocked out gene copy contributes to the breaking of dormancy / promotion of germination under those conditions.

All GA3ox functional knock-out mutants used in these assays were obtained from the Tai-ping Sun laboratory (Duke University) and were created from the Columbia background ecotype. Lines assayed included each single gene copy knockout (*ga3ox1-* through *ga3ox4-)* two double knockouts (*ga3ox1/2- , ga3ox2/3-)* and one triple knockout (*ga3ox1/2/3-)*. Phytochrome mutants were generated from the Landberg erecta (Ler) background in the Whitlam/Sharrock lab and from the Col background in the Quail/Meng lab. Col mutant lines assayed were $phyB_{19}$, $phyB_{9}$, $phyC_{1}$, $phyC_{2}$, $phyD_{201}$, $phyE_{201}$ (where the subscript number represents the specific allele that was knocked out) and Ler mutant lines assayed include*, phyB, phyD* and *phyE.*

Four replicates of each mutant genotype and their wild-type ecotype were planted and stratified (a cold, dark imbibition treatment that reflects winter conditions to break seed dormancy) at 4C for 5 days. Pots were placed in the Duke Phytotron growth chambers at 22C with 14h light days to reflect typical spring maturation conditions. Once siliques were matured, seeds were harvested and dried at room temperature under a hood. 12 seeds per line were then placed in petri plates containing 0.5% agar with 12 replicate plates per line per temperature treatment (i.e. - 144 seeds per genotype per treatment).

Each plate was exposed to four temperature treatments in order to investigate each PHY and GA3ox's role in dormancy breakage / induction in response to combinations of stratification temperatures (recently imbibed seeds put into the dark) and germination temperatures (in white light incubation chambers). This was tested by comparing the germination responses of each mutant genotype to its background ecotype after 10 days in the light after the following four combinations of treatments: '4-10' (5

dark days at 4C, put in light at 10C), ‘4-22’ (5 dark days at 4C, put in light at 22C), ’31-22’ (5 dark days at 31C, put in light at 22C), ’22-22’ (5 dark days at 22C, put in light at 22C). Following each 5 -day dark stratification treatment, plates were transferred to Percival germination incubator chambers with 12-hr photoperiod days and germination response were recorded after 10 days in the light. To test the functional diversifications in response to germination temperature, the germination proportions of the mutants to their background ecotype in the ‘4-10’ versus ‘4-22’ treatments were compared. To test the diversifications in response to dormancy-breaking temperatures, ‘4-22’ versus ’22-‘22’ germination responses were compared. Finally to test the diversifications after a dormancy-inducing treatment, ’31-22’ versus ’22-22’ germination responses were compared.

Germination proportion of each genotype (or line) was calculated as the number of viable seeds per plate divided by total number of germinates. PHY and GA3ox knockouts mutants with significantly reduced or raised germination proportions compared to its background ecotype supports their role in either promoting or inhibiting germination (by either breaking or inducing dormancy, respectively) under a given combination of temperature conditions. To test for significant differences in germination responses among genotypes, a series of ANOVAs were conducted and separate non-parametric Tukey-Kramer tests were performed within each combination of temperature treatments. We also performed separate two-way ANOVAs for each mutant-background pair to test for significant differences between the mutant genotypes and the background ecotype in response to each temperature treatment. Significant treatment-by-genotype interaction (ie - treatment x genotype) from these ANOVAs indicates significant

differences between the mutant genotype and its wild-type, supporting the role of that functionally disrupted (i.e. – 'knocked-out') gene copy in contributing to germination in response to this temperature treatment.

Finally, to test whether PHY and GA3ox copies contribute to germination non-additively (i.e.– are functionally redundant) with other PHY / GA3ox copies we compared multiple knockout mutants germination responses to single knock-outs germination response using a two-way ANOVA (e.g. - effects = GA3ox1 + GA3ox2 + GA3ox1xGA3ox2) where functional allele coded as [1] and non-functional as [0].

1.2.1 Effect of after-ripening on the diversification GA3ox copies following dormancy-inducing imbibition temperatures

This assay was performed with the same seeds harvested for Assay 1 in order to investigate the effect that after-ripening has on the functional diversification and GA3ox copies in promoting germination when seeds don't experience a dormancy-breaking (4C) stratification treatment. A period of dry-after ripening is known to reduce the dormancy level of seeds so only the dormancy-inducing '31→22' temperature treatment (5 dark days at 31C, put in light at 22C) and '22→22' treatment (5 dark days at 22C, put in light at 22C) were tested. We expected to find higher germination responses to both of these treatments than the responses to these treatments from assay 1 since after-ripening breaks dormancy and the seeds used in assay 1 were fresh. To test the effect of after-ripening in general and to test whether different copies' diversified roles are affected differently by after-ripening, the same seeds from assay 1 were used in this assay. Thus, the same germination assay methods and statistical comparisons to test for significant

differences and non-additive interactions among genotypes and between mutants and the wild-type used in assay 1 were used in this assay as well.

1.2.3 Diversification of GA3ox in response to a range of germination temperatures following dormancy-breaking/inducing conditions

Finally, the diversification of GA3ox copies role in germination was characterized over a *range* of temperature conditions following either a dormancy-inducing (31C) or dormancy-breaking (4C) stratification treatment. A 22C stratification was also used to compare the effects of these dormancy-contributing conditions on germination responses. Following each stratification treatment seeds were put into the light at 8C, 16C, 22C, and 31C and germination proportions were assayed after 10days. The same plating methods and Percival germination chambers described in the previous two assays were used in this assay as well. Seeds used in this assay were one-month after-ripened so were more after-ripened then the 'fresh' seeds used in assay 1 but less after-ripened than those used in assay 2.

To test for significant differences among the genotypes in response to stratification and germination temperatures, separate ANOVAs were conducted and non-parametric Tukey-Kramer tests were performed. First, a full model with stratification, germination temperature, and genotype was conducted with all of the lines to test for significant interactions between the combination of temperature conditions and the genotypes. Next, to test for the diversification of GA3ox copies in response to germination temperature, separate two-way ANOVAs were conducted within each stratification treatment (4C, 22C, 31C) and significant differences between mutant

genotypes and background ecotype germination proportions were confirmed using non-parametric Tukey-Kramer tests. Then, to test the effect of stratification on the diversification of GA3ox copies, separate two-way ANOVAs were performed within each germination temperature (8,16,22, and 31C) and significant differences between mutants and the background-ecotype were confirmed using non-parametric Tukey-Kramer tests. Finally to test for the functional diversification of GA3ox copies in response to combinations of imbibition (dark) and germination (light) temperature cues, separate ANOVAs were performed within each combination of stratification and germination temperature and significant differences between genotypes were tested using non-parametric Tukey-Kramer tests.

1.3 Results

1.3.1 Assay 1*: Functional Diversification of PHY & GA3ox copies in response to germination temperatures and dormancy-breaking /inducing imbibition temperatures*

Only the disruption of PHYE (on the Ler background) and PHYB (on the Col background) had significantly different germination responses to germination temperature following a dormancy-breaking (4C) stratification. Disrupting PHYE (on Ler) significantly reduced germination at 10C but not 22C following a 4C stratification (Fig. 1) suggesting that PHYE contributes to germination in cold temperatures following dormancy-breaking stratification. $phyB_{19}$ and $phyB_{9}$ had significantly lower germination

proportions than Col ecotype at both 22C and 10C following a cold stratification treatment (Fig. 2), suggesting that PHYB contributes to germination in cold and "neutral" (22C) temperature conditions following a dormancy-breaking stratification treatment.

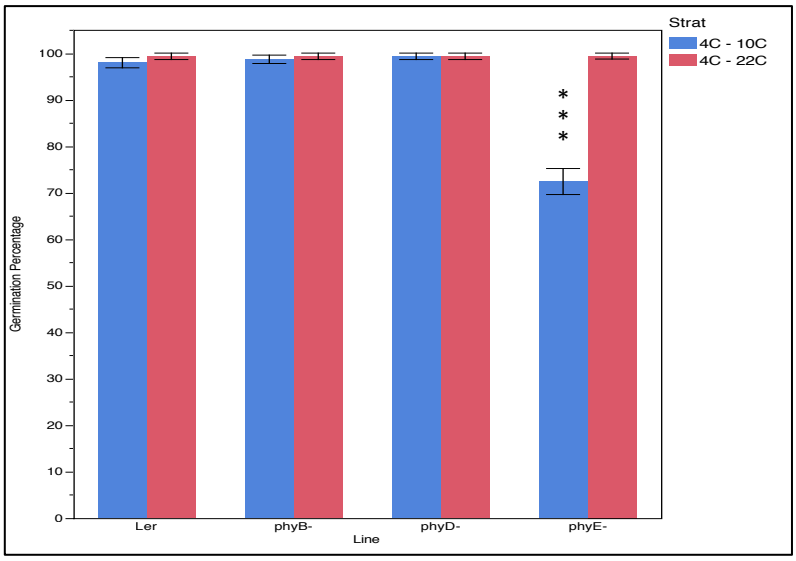

Fig. 1 Germination percentages of Landsberg *erecta* (Ler) lines after 10 days in the light at 10C and 22C following the same 5 day stratification treatment at 4C. Signficant genotype x stratification interaction effect calculated in a two-way ANOVA to test whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type. * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

Seeds exposed to a 4C stratification treatment before being put into the light at 22C had significantly higher germination proportions than those exposed to a 22C stratification. Thus, a cold-temperature stratification treatment significantly lowers the levels of dormancy across genotypes when germinating at 22C (Fig. 2, 3,4).

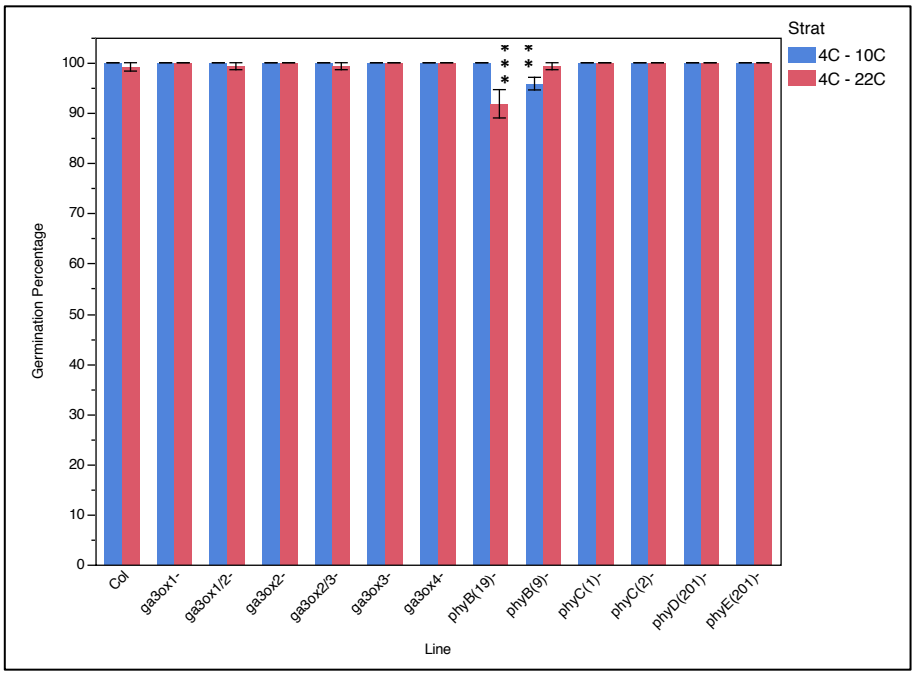

Fig. 2 Germination percentages of Columbia (Col) lines after 10 days in the light at 10C and 22C following the same 5 day stratification treatment at 4C. Signficant differences between mutant genotype and wild-type calculated using Tukey-Kramer tests performed in separate two-way ANOVAs within eac combination of stratification treatments and germination temperatures. * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

All of the genotypes had significantly lower germination proportions following a 31C stratification than a 22C stratification, confirming the ability of warm-stratification temperatures to induce dormancy. On the Ler ecotype background, disruption of PHYB resulted in significantly reduced germination below the wild-type in 22C germination temperature after seeds were exposed to a 22C stratification suggesting PHYB's role in contributing to germination at 22C when seeds don't experience a dormancy-inducing stratification treatment (Fig. 3). Disruption of PHYD significantly reduced germination at 22C after seeds were exposed to a 31C stratification temperature suggesting PHYD's role in promoting germination following a warm, dormancy-inducing stratification treatment (Fig. 3).

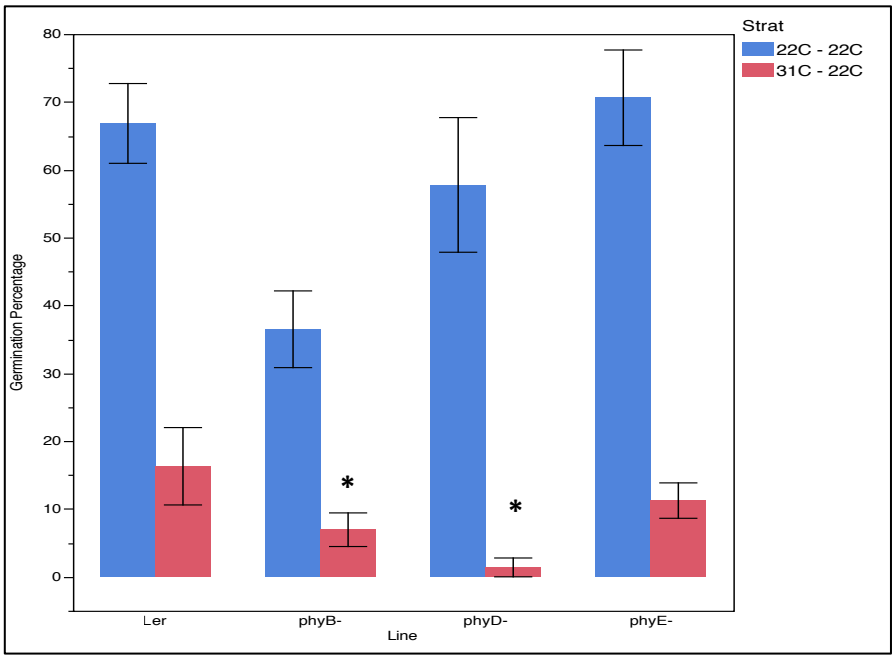

Fig. 3 Germination percentages of Ler lines after 10 days in the light at 22C following warm and neutral stratification treatments. Signficant genotype x stratification interaction effect calculated in a two-way ANOVA to test whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type. * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

On the Col background, *ga3ox1*- and *ga3ox4*- had significantly higher germination proportions than the wild-type at 22C following a 31C and 22C stratification (Fig. 4). Thus, GA3ox1and GA3ox4 appear to suppress germination (or promote dormancy maintenance) at 22C if seeds don't experience a dormancy-breaking (cold) stratification treatment.

Interestingly however, disrupting GA3ox1 and GA3ox3 on a GA3ox2 deficient mutant background (ie – *ga3ox1/2*- and *ga3ox2/3*-) significantly reduced germination below either *ga3ox1*- or *ga3ox3*- (Fig.4). This would suggest that GA3ox1 and GA3ox3 somehow interact with GA3ox2 to promote germination - a previously undocumented role for GA3ox3 in the regulation of temperature-dependent seed germination. Disruption of GA3ox2 alone however did not significantly reduce germination below the wild-type after either a 31C or 22C stratification.

In summary, GA3ox1 appears to inhibit germination following warm and neutral stratification (since disrupting it significantly raises germination above the wild-type) however disrupting it with GA3ox2 reduces germination. Moreover, disrupting GA3ox2 and GA3ox3 alone didn't significantly affect germination; however *ga3ox2/3-* did significantly reduce germination below either of their single mutant germination proportions (Table 1). These findings suggests a complex and intriguing interaction between all three of these GA3ox copies, possibly involving complicated negative feedback mechanisms, hypothesized in greater detail in the 'Discussion' section.

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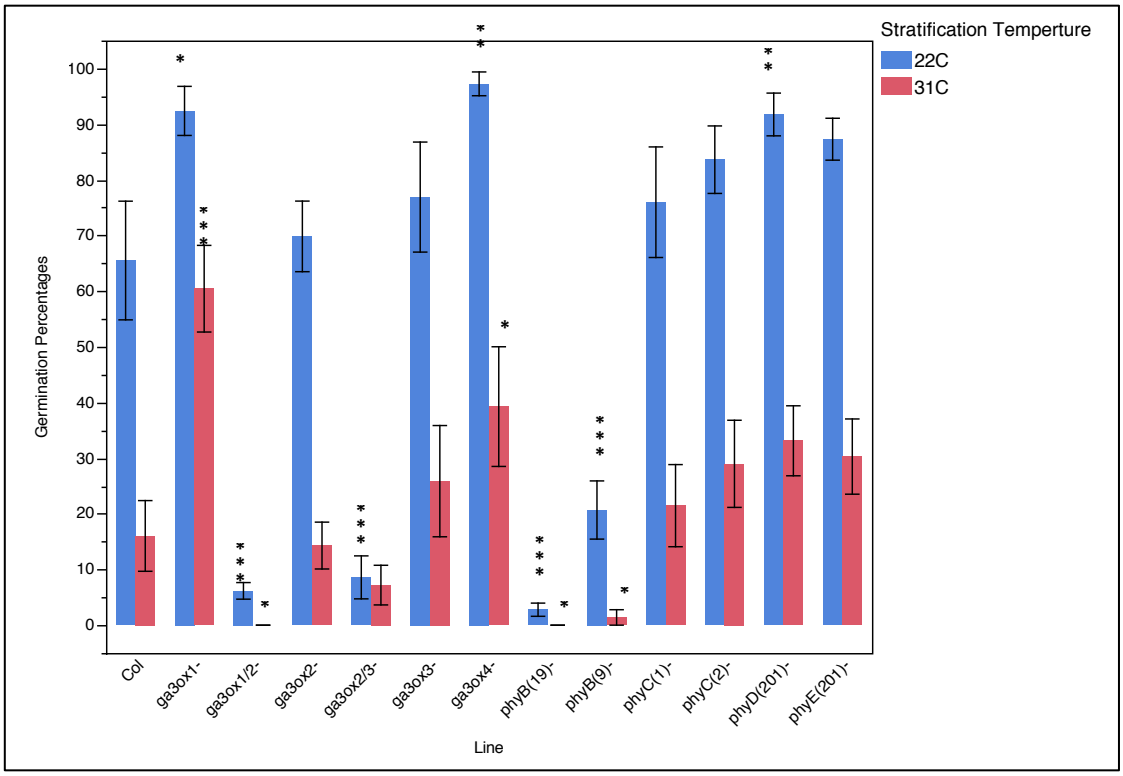

Fig. 4 Germination percentages of Col lines after 10 days in the light at 22C following warm and neutral stratification treatments. Signficant genotype x stratification interaction effect calculated in a two-way ANOVA to test whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type. * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

Table 1. Tests for nonadditive interactions between GA3ox alleles in each combination of temperature treatments

Interaction	4C – 10C	4C – 22C	31C – 22C	22C – 22C
ga3ox1 x ga3ox2	n/a	1.9836	29.0702***	44.5367***
ga3ox2 x ga3ox3	n/a	1.9836	1.6836	17.9757***

Note: F ratios given from two-way ANOVA (effects = GA3ox1 + GA3ox2 + GA3ox1xGA3ox2) where functional allele coded as [1] and non functional as [0]. Tests for nonadditive interactions between GA3ox1 / GA3ox2 and GA3ox2 / GA3ox3. F ratios couldn't be calculated for 4C – 10C treatment because MS_{error} was 0 (ie – no variance).

1.3.2 Assay 2*: Effect of After-ripening on GA3ox's Diversified Roles*

In 6-month dry-after seeds from assay 1, the back-ground ecotype (Col) had significantly higher germination proportions after a 22C and 31C stratification than it did when these seeds were fresh (Fig. 5). Thus, a 6-month period of after-ripening did significantly reduce dormancy levels in the background ecotype's seeds.

In contrast to fresh seeds, *ga3ox2-* had significantly reduced germination below the wild-type ecotype after both warm (31C) and neutral (22C) stratifications (Fig. 5) Thus, GA3ox2 appears to be an important contributor to germination at 22C in after-ripened seeds following warm and neutral imbibition temperatures (Fig. 3). Disruption of GA3ox1 alone did not significantly influence germination at 22C after either a 31C or 22C stratification. Thus, GA3ox1's possible role in inhibiting germination at 22C (as seen in the fresh seeds in assay 1) was not visible in dry after-ripened seeds, in part because the wild-type was completely non-dormant even after a warm stratification (i.e. – Col seeds had nearly 100% germination).

Disrupting GA3ox1 on a GA3ox2 deficient mutant significantly reduced germination proportions below *ga3ox2-* proportions at 22C following a 22C stratification (Fig. 5, Table 2). Thus, GA3ox1 appears to contribute to germination in dry

after-ripened seeds not experiencing a dormancy-inducing/breaking stratification treatment but it does so redundantly with GA3ox2. This contribution was confirmed by the significant non-additive interactions of GA3ox1 and GA3ox2 in a '22→22' treatment (Table 2). This was consistent with the non-additive interaction found between these two copies in fresh seeds (Table 1).

In summary, GA3ox2 appears to be the most important contributor to germination at 22C in dry after-ripened seeds following either a warm or neutral stratification treatment, while GA3ox1 appears to contribute redundantly and non-additively with GA3ox2 in after-ripened seeds only after a 22C stratification treatment. GA3ox1 alone however is not a significant contributor to germination at 22C in dry after ripened seeds exposed to either a neutral or warm stratification treatment (Fig. 5). GA3ox1 inhibiting contribution seen in fresh seeds however could not have been apparent however since the Col wild-type was completely non-dormant (ie – nearly 100% of Col seeds germinated).

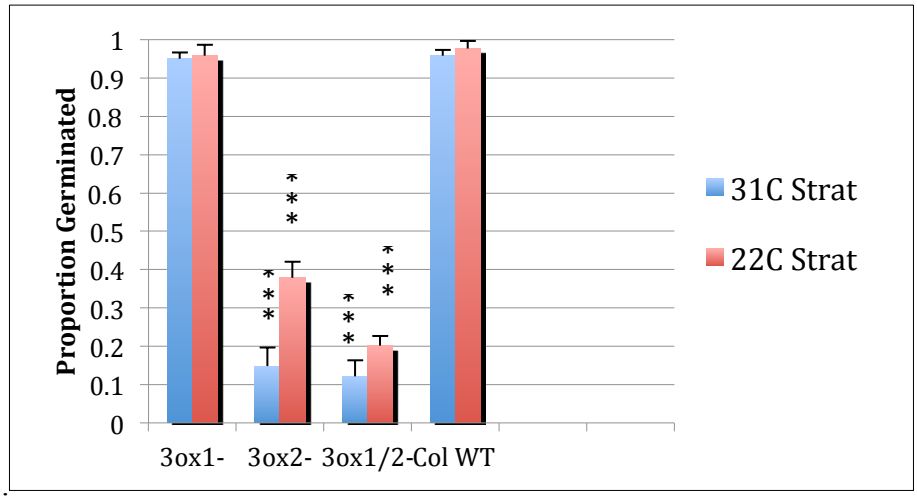

Fig. 5 Germination proportions of GA3ox1 and GA3ox 2 at 22C after 10 days in light following a 5 day stratification at either 31C or 22C. Significant genotype x stratification interaction effect calculated in a two-way ANOVA to test whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type. * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

Table 2. Tests for significant mean differences between mutant and wild-type and interaction between GA3ox alleles.

Mutants vs. Col	**31C - 22C**	**22C - 22C**	**Genotype x stratification**
ga3ox1-	0.0496	0.678	0.4095
ga3ox2-	318.82***	129.57***	9.31**
ga3ox1/2-	685.83***	283***	1.2
ga3ox1 x ga3ox2	0.1075	5.2941*	n/a

Note: F ratios are given in the table above from ANOVA's which were done by JMP 10 software. The last column gives the F ratios from genotype x stratification interaction effect calculated in a two-way ANOVA (tests whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type). * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

In one-year after-ripened seeds[1], there was no difference in the germination proportions of GA3ox mutants to the background ecotype at 22C following a 22C stratification. Following a 31C stratification however, *ga3ox2-* significantly reduced germination below the wild-type at 22C (Fig. 7). Thus, GA3ox2 appears to be an important contributor to germination at 22C in after-ripened seeds following a dormancy-inducing stratification treatment (consistent with the finding of GA3ox2's role in 6-month after-ripened seeds as well, Fig. 3). Disruption of GA3ox3 alone did not significantly reduce germination below the wild-type after a 31C stratification, but *ga3ox2/3-* did have a significantly lower germination proportion than *ga3ox2-* (Fig. 7) There was also a significant non-additive interaction between GA3ox2 and GA3ox3 suggesting these copies contribute to germination redundantly (Table 3).

[2] Dr. Kathleen Donohue was responsible for creating the following model's parameters and equations. I was responsible for coming up with comparisons to test the effect of environmental

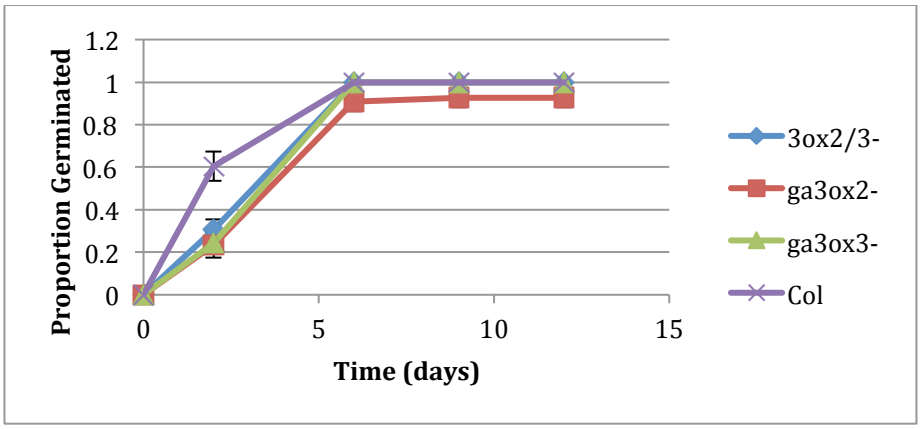

Fig. 6 Germination proportions at 22C after 12 days in light following neutral (22C) stratification. No significant differences. See Table 3 below

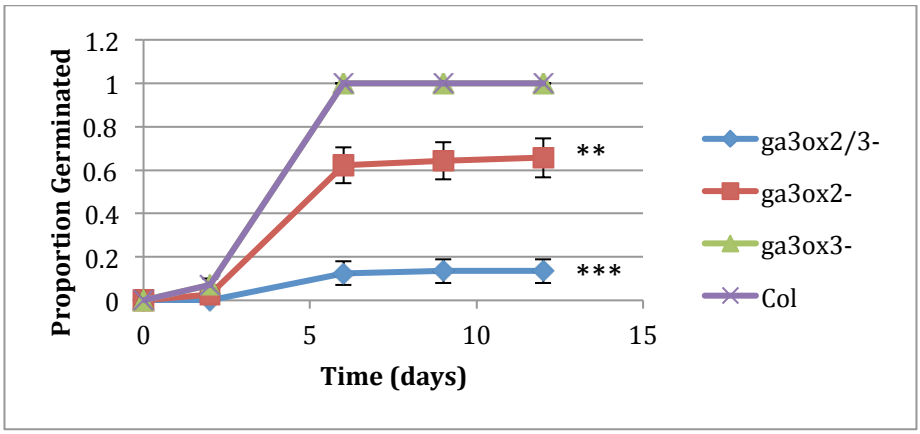

Fig 7. Germination proportions at 22C after 12 days in light following warm (31C) stratification treatment. * P < 0.05; ** P < 0.001; *** P < 0.0001

Table 3. Tests for significant differences between mutant and wild-types and interaction effect between GA3ox-mutant alleles.

Mutants vs. Col	31C - 22C	22C - 22C	Genotype x stratification
ga3ox2-	13.7763**	2.8837	6.1917*
ga3ox3-	3.1966		3.1966
ga3ox2/3-	246.4711***	1.000	276.9897***
ga3ox2 x ga3ox3	24.2754***	2.4979	n/a

Note: F ratios are given in the table above from ANOVAs which were done by JMP 10 software. The last column gives the F ratios from genotype x stratification interaction effect calculated in a two-way ANOVA (tests whether the combination of genotype and stratification significantly influences each mutant differently than it influences the wild-type). Blank indicates F ratio could not be calculated because Mean Square Error (MS_{error}) was 0. * P < 0.05; ** P < 0.001; *** P < 0.0001

1.3.3 Assay 3*: Diversification of GA3ox copies in response to a range of germination temperatures*

Effect of dormancy-breaking imbibition on the diversification of GA3ox copies in response to germination temperatures

After a 4C stratification, the disruption of GA3ox2 significantly reduced germination proportions below the background ecotype in each of the germination temperature conditions. This effect was most pronounced when seeds germinated at 31C. Thus, GA3ox2 appears to be an important contributor to germination in a range of temperatures, especially warm, following a dormancy-breaking stratification treatment (Fig. 8)

The disruption of GA3ox1 did not significantly reduce germination in response to germination temperature below the background ecotype after a 4C stratification. Disruption of GA3ox1 along with GA3ox2 however restored germination above the *ga3ox2-* proportions, suggesting GA3ox1 plays a role in inhibiting germination in response to cold imbibition temperatures. *Ga3ox1- and ga3ox1/2-* germination proportions varied most significantly when seeds germinated at 31C suggesting that GA3ox1 role in inhibiting germination after a dormancy-breaking stratification is most pronounced under warm germination temperatures (Fig. 8)

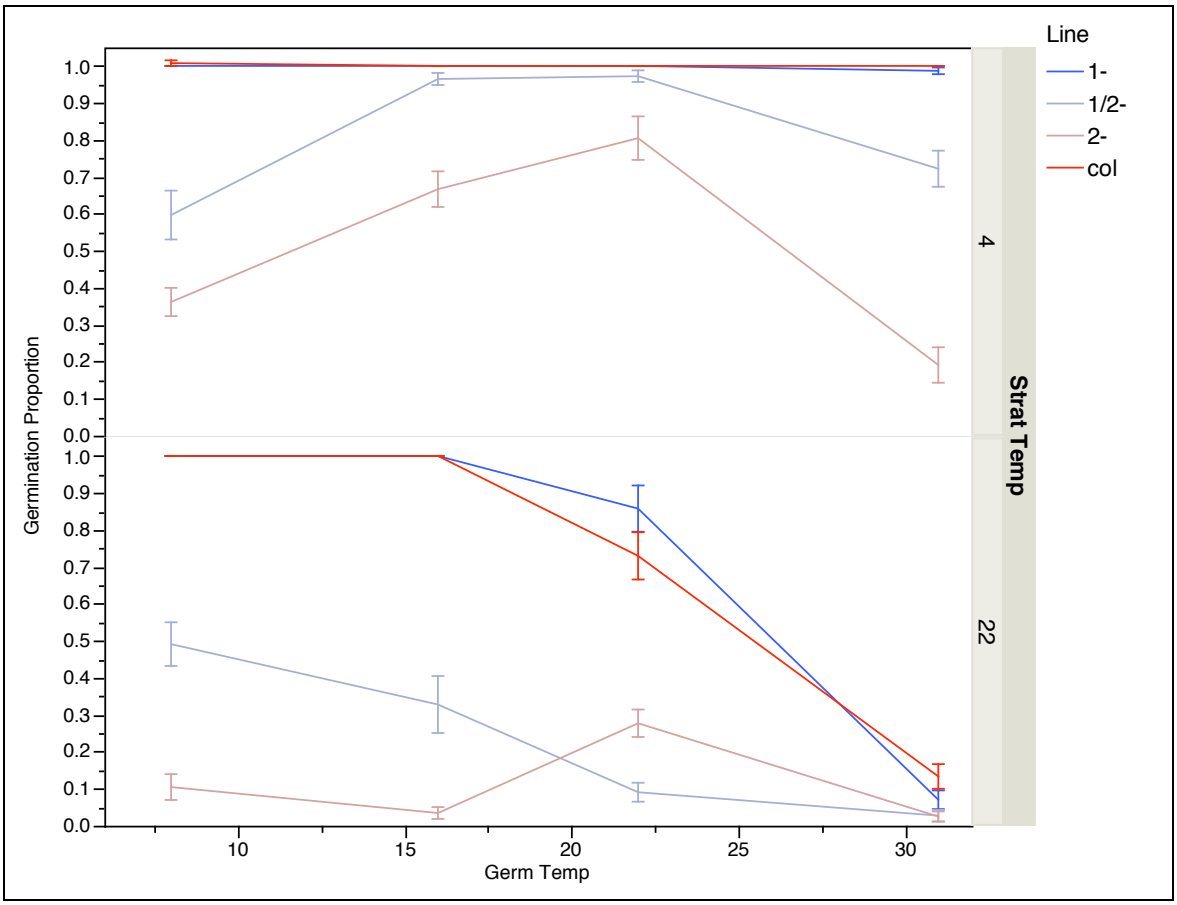

Fig. 8 Germination proportions of GA3ox knockout mutants and Col wild-type after 10 days in the light following a 5 day dark-stratification treatment at 4C. Error bars constructed using 1 standard deviation from the mean. (Note – *ga3ox2-. ga3ox3-, and ga3ox2/3-* mutants were excluded from this stratification treatment).

Diversification of GA3ox copies in response to germination temperatures without dormancy-breaking/inducing imbibition

Following a 22C stratification, disrupting GA3ox2 once again significantly reduced germination below the wild-type in response to germination temperature (Fig. 9). Unlike in the 4C stratification however, this reduction was least significant when seeds germinated at 31C. Disrupting GA3ox3 significantly raised germination at 22C following a 22C stratification above the wild-type suggesting that GA3ox3 may inhibit germination under these conditions (Fig. 9). Disrupting GA3ox3 in combination with GA3ox2 significantly significantly reduced germination below the wild-type at 16C

and 22C. *ga3ox2/3-* germination proportion was also significantly higher than *ga3ox2-* at 8C and 16C but not 22C and 31C (Fig. 9). Finally disrupting GA3ox1 did not significantly effect germination at any germination temperature following a 22C stratification. Disrupting it with GAox2 however significantly restored germination above *ga3ox2-* at 8C and 16C but not at 22C and 31C. Thus, GA3ox1 and GA3ox3 appear to be inhibiting germination at 8C and 16C but promoting it redundantly with GA3ox2 at 22C. These possible 'inhibitory' contributions of GA3ox1 and GA3ox3 aren't observed at 8C and 16C in the single knockout mutants since the background ecotype (Col) had such high germination proportions under these conditions (Fig. 9).

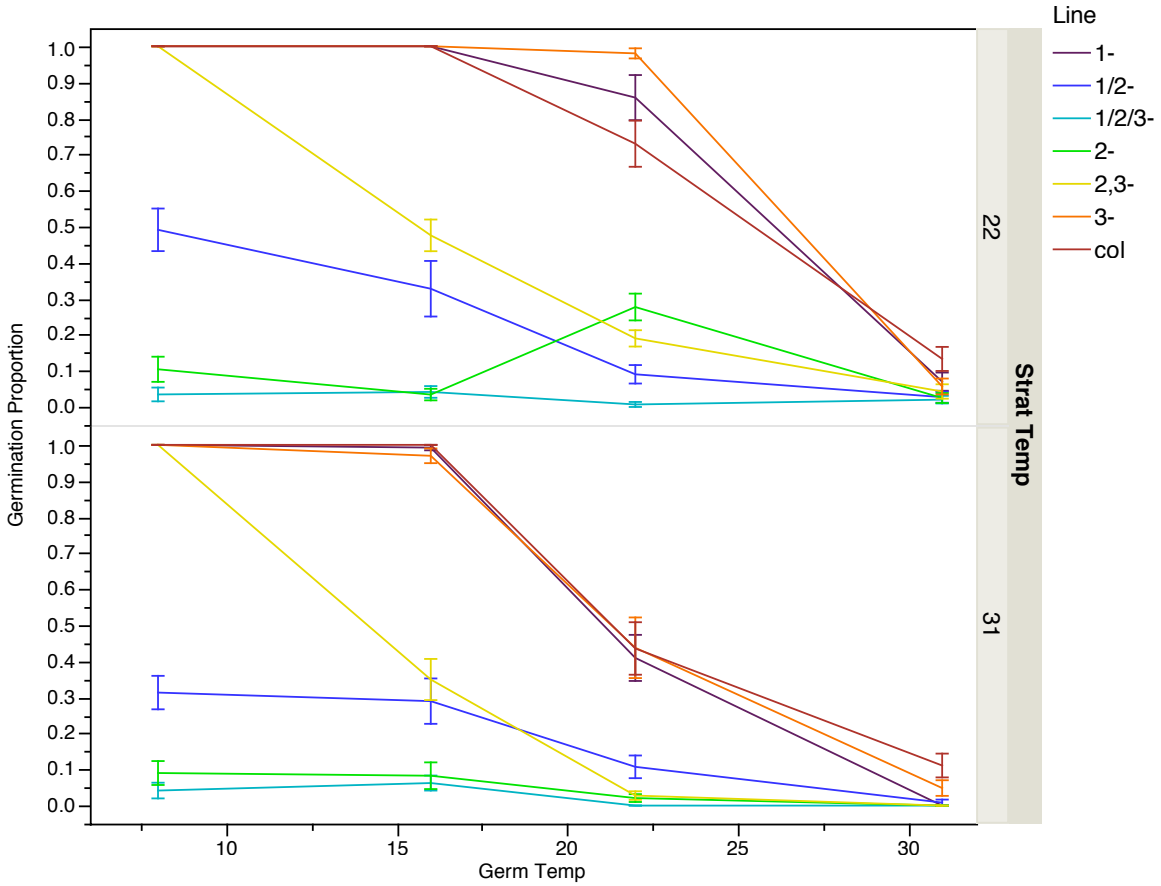

Fig. 9 Germination proportions of GA3ox knockout mutants and Col wild-type after 10 days in the light following a 5 day dark-stratification treatment at either 22C or 31C. Error bars constructed using 1 standard deviation from the mean.

Diversification of GA3ox copies in response to germination temperatures with dormancy-inducing imbibition

After a 31C stratification, disrupting GA3ox2 once again reduced germination in all of the germination temperature conditions (Fig. 9) Disrupting GA3ox1 and GA3ox3 in combination with GA3ox2 also restored germination above the *ga3o2-* proportion at 8C and 16C but not 22C or 31C (consistent with their germination responses to temperature after the 22C stratification) (Fig. 9).

In summary, disruption of GA3ox2 did significantly reduce germination below the wild-type in all of the germination temperature conditions and after each stratification treatment. Thus, GA3ox2 appears to be the most important contributor to germination in a range of temperature conditions after either a dormancy-breaking or dormancy-inducing stratification treatment (Fig. 8,9; Table 4). Disrupting GA3ox1 and GA3ox3 alone did not significantly contribute to germination in any combination of temperature conditions, except for *ga3ox3-* increased proportion at 22C following a 22C stratification (Table 4). Following a non-dormancy breaking stratification, disrupting GA3ox1 or GA3ox3 in combination with GA3ox2 however, restored germination above *ga3ox2-* at 8C and 16C but not 22C or 31C (Fig. 9). Thus, GA3ox1 and GA3ox3 appear to be inhibiting germination at 8C and 16C following either a 22C or 31C stratification. These possible 'inhibitory' contributions of GA3ox1 and GA3ox3 aren't observed at 8C and 16C in the single knockout mutants since the background ecotype such high germination proportions in these conditions.

In response to stratification temperature, the Col wild-type's germination proportions were statistically different when germinating at 22C and 31C but not 8C or 16C. Thus, regardless of stratification, the background ecotype was non-dormant when germinating at 8C and 16C contributing to the inability to see GA3ox1 and GA3ox3's suspected inhibitory functions under these conditions. At 22C, Col's germination proportion was significantly higher in response to a 22C stratification than a 31C stratification. Thus, warmer stratification temperatures appeared to decrease the optimal temperature of germination in the background ecotype.

Table 4. Tests for significant differences between mutants and background (Col) genotype in each combination of stratification and germination temperatures

Mutant vs. Col	**8C**	**16C**	**22C**	**31C**
4C Stratification				
Ga3ox1-	-.0076	1.1e-16	0	-0.0134
Ga3ox2-	-0.646***	-0.333***	-0.027**	-0.8088***
Ga3ox1/2-	-0.411***	-0.354	-0.195	-0.1478***
22C Stratification				
Ga3ox1-	0	0.1482	0.1278	-0.063
Ga3ox2-	-0.8955***	-0.9658***	-0.4527***	-0.1084*
Ga3ox1/2-	-0.5089***	-0.6722***	-0.6342***	-0.106*
Ga3ox3-	0	0	0.2504**	-0.0764
Ga3ox2/3-	0	-0.5243***	-0.5396***	-0.0903
Ga3ox1/2/3-	-0.9653***	-0.9583***	-0.7229***	-0.1129*
31C Stratification				
Ga3ox1-	2.2e-16	-.0076	-.0262	-0.0111
Ga3ox2-	-0.9097 ***	-0.9172***	-0.4147***	-0.1211***
Ga3ox1/2-	-0.6867***	-0.7105***	-0.3281***	-0.1028**
Ga3ox3-	2.2e-16	-0.0303	0.0019	-0.0663
Ga3ox2/3-	4.2e-16	-0.6505***	-0.4147***	-0.1026**
Ga3ox1/2/3-	-0.9583***	-0.9375***	-0.4356***	-0.1431***

Note: Separate ANOVAs were performed in each combination of stratification and germination temperatures and significant differences were tested with non-parametric Tukey-Kramer tests. Values represent the difference in the LeastSquare means of ecotype and mutant genotype. Negative values represent mutants with lower germination responses than the wild-type * $P < 0.05$** $P < 0.001$*** $P < 0.0001$

1.4 Discussion

The germination assays conducted in the first part of this research project indicate the functional divergence of various duplicated phytochrome and gibberellin3-oxdiase gene copies with respect to temperature-dependent seed germination (Table 5). We found that the temperature that seeds are exposed to both in the dark (through stratification treatments) and the light (during germination conditions) influence the degree to which different PHY and GA3ox copies contribute (either redundantly or divergently) to germination. We also found evidence to support that some of these gene copies may not only contribute to germination but also inhibit germination (or maintain dormancy) in response to certain combinations of temperature conditions seasonal environments. A summary of each PHY and GA3ox role during temperature-dependent seed germination is summarized in Table 5.

Table 5. Summary of PHY and GA3ox contributing role to germination in response to imbibition and germination temperatures

Gene (Ecotype)	**Temperature Conditions with significant contribution (Light → Dark)**	**Summary of Contribution to Germination**
Phytochromes (Ler)		
PHYB	22C → 22C	Promotes when seeds don't experience dormancy breaking/inducing imbibition
PHYD	31C → 22C	Necessary after dormancy-inducing imbibition
PHYE	4C → 10C	Required in cold temperatures after dormancy-breaking imbibition
Phytochromes (Col)		
PHYB	22C or 31C → 22C 4C → 10C or 22C	Contributes in range of temperature conditions after both dormancy-breaking /inducing imbibition
PHYC	-	-
PHYD	22C → 22C	Inhibits if dormancy not broken/induced by imbibition
PHYE	-	-

Gibberellin 3-oxidases (Col)		
GA3ox1	22C or 31C → 22C	Contributes redundantly with GA3ox2 if seeds don't experience dormancy-breaking imbibition
	22C or 31C → 8C – 16C	Inhibits germination in cool conditions when dormancy not broken by imbibition.
GA3ox2	4C → 8 – 31C 22C → 8 – 22C 31C → 8 – 22C	Necessary in wide range of imbibition/germination temperatures, particularly in after-ripened seeds
GA3ox3	22C or 31C → 22C	Contributes redundantly with GA3ox2 (may explain *ga3ox1*- increased response if GA3ox1 inhibiting GA3ox3)
	22C or 31C → 8C – 16C	Inhibits germination in cool conditions when dormancy not broken by imbibition
GA3ox4	22C or 31C → 22C	Inhibits if dormancy not broken by cold imbibition

Note: Green shaded blocks indicate gene copy's suspected role in promoting germination while red-shaded blocks indicate suspected role in inhibiting germination. No observed role in contributing to germination indicated by blank white blocks.

Phytochrome Diversification

The first germination assay confirmed the previously recorded roles of PHYB, PHYD and PHYE in promoting germination in response to temperature. On the Ler background, PHYB was necessary to promote germination in neutral (22C) germination temperature conditions after a neutral stratification treatment, PHYD was necessary for germination in neutral temperatures following a warm stratification treatment, and PHYE was necessary for germination in cold temperature conditions after a cold, dormancy-breaking stratification treatment (Heschel et al. 2008). Interestingly however these environmentally divergent contributions to germination of PHY copies derived from the Ler ecotype were not observed in the PHY mutants derived from the Columbia background (with the exception of PHYB's role in promoting germination in neutral temperatures without a cold stratification treatment). The Columbia (Col) ecotype is known to be more dormant than Lansberg erecta (Ler) ecotype however. This might explain, for example, why we only notice PHYB's contribution to

germination following a dormancy-breaking (4C) treatment in the Col mutant lines but not the Ler lines. Ultimately, it is unlikely that different phytochrome copies have actually evolved different functionally divergent roles in the different *A. thaliana* ecotypes during temperature-dependent germination. Rather their diversified roles are either masked or highlighted by the background's ecotype response to a given combination of temperature conditions.

GA3ox Diversification

In the first assay, none of the GA3ox mutants had significantly lower germination proportions than the wild-type following a 4C stratification treatment. The wild-type (Col) was completely non-dormant however following this 4C stratification treatment. Thus, certain GA3ox's may still contribute to germination after a cold stratification but more than two copies must be knocked out to see a reduced germination response. In other words, cold-induced germination may not be promoted exclusively through just two GA3ox copies in fresh seeds.

This finding was inconsistent with assay 3 where disruption of GA3ox2 did significantly reduce germination in a range of temperature conditions following a cold stratification treatment. The seeds in assay 3 were slightly more after-ripened than those in assay 1 however. Thus, GA3ox2's role in promoting germination following a dormancy-breaking stratification may be dependent on the period of dry after-ripening.

In assay 2, GA3ox2 appeared to have a significant contribution to germination at 22C in 6-month and one-year after-ripened seeds. Unlike the fresh seeds from assay 1, disruption of GA3ox2 significantly reduced germination below the wild-type at 22C

following both 22C and 31C stratifications in after-ripened seeds. Given that GA3ox2 is not subject to any negative feedback like GA3ox1, it is plausible that a functional GA3ox2 copy is necessary in order for a seed to accumulate enough bioactive GA over the period of dry after-ripening to overcome primary dormancy and/or break secondary dormancy induced by warm imbibition temperatures (Yamaguchi 2008).

When fresh seeds did not experience a dormancy-breaking stratification, disruption of GA3ox1 significantly increased germination above the background ecotype, particularly after a warm stratification treatment. This suggests that GA3ox1 may inhibit germination (or help maintain dormancy) after a dormancy-inducing temperature treatment. Disruption of GA3ox1 on a disrupted GA3ox2 background however, significantly lowered germination below either single knockout mutant after both a 22C and 31C stratification. Thus, GA3ox1 and GA3ox2 may actually contribute redundantly to germination. This peculiar difference in the single versus double knockout mutants of GA3ox1 may be explained by its possible interactions with GA3ox3.

Disruption GA3ox3 alone did not significantly affect germination in response to stratification temperature in assay 1. Similar to GA3ox1 however, disrupting it in combination with GA3ox2 (*ga3ox2/3-)* significantly reduced germination below both the wild-type's and *ga3ox2-'s* germination proportions. This suggests that GA3ox3 may be promoting germination redundantly with GA3ox2, a previously unknown role of GA3ox3 in temperature dependent seed germination. If GA3ox1 is inhibiting GA3ox3 expression however, then GA3ox3 contribution to germination (alongside GA3ox2) may explain the increased germination proportion of *ga3ox1* – above the

wild-type in assay 1. Ultimately, the disruption ofGA3ox1 or GA3ox3 only results in reduced germination when GA3ox2 is knocked out as well. This suggests that GA3ox2 copy is the dominant 'contributor' to germination under these conditions and is able to produce enough bioactive GA to promote germination even when fresh seeds don't receive a dormancy-breaking (cold) stratification.

Consistent with fresh seeds, both GA3ox1 and GA3ox3 appear to contribute redundantly to germination with GA3ox2 in after-ripened seeds exposed to warm stratification treatments. This redundant contribution is indicated by the significant non-additive interactions between GA3ox1 / GA3ox3 and GA3ox2 observed in both assays 1 and 2 (Table 1 and 2). In summary, the double mutants significantly reduced germination in fresh and after-ripened at 22C when seeds were not exposed to a dormancy-breaking stratification. Only in after-ripened seeds, however, did the disruption of GA3ox2 did significantly reduce germination in response to these conditions. Thus, GA3ox2 may be necessary for germination at 22C in after-ripened seeds but not fresh seeds if dormancy isn't broken by a cold imbibition treatment.

Finally, in assay 1, disruption of GA3ox1 significantly raised germination above the wild-type after 31C and 22C stratifications but disruption of GA3ox3 did not. Thus, GA3ox1 may be the dominant 'suppressor' germination under these conditions. This is also supported by the significantly reduced *ga3ox2/3-* germination proportion below *ga3ox1/2-* in dry-after ripened seeds exposed to warm stratification temperatures (Fig. 5, Fig. 7). The GA3ox2/3 mutants were also six months *more* after ripened than the GA3ox1/2 mutants, further supporting GA3ox1's role as the major contributor to dormancy in dry-after ripened seeds.

In assay 3, *ga3ox1/2-* and *ga3ox2/3-* had lower germination proportions than *ga3ox2-* at 22C following a non-dormancy breaking stratification (consistent with assays 1 and 2). When GA3ox mutants germinated at 8C and 16C following these stratification treatments however, *ga3ox1/2-* and *ga3ox2/3-* had significantly higher germination proportions than *ga3ox2-*. This suggests that when dormancy is not broken by a cold stratification treatment, GA3ox1 and GA3ox3 inhibit germination in response to cold germination temperatures but may promote germination (redundantly with GA3ox2) in response to high germination temperatures.

Again, GA3ox1 and GA3ox3 role in inhibiting and/or promoting germination may be obscured by the fact that GA3ox2 appears to be the most important contributor to germination in response to a range of light temperature conditions (3C → 31C) following either dormancy-breaking or dormancy-inducing stratification treatments (Table 4, Fig. 9). Thus, in most temperature conditions, the effect of GA3ox1 and GA3ox3 on germination is only observed when GA3ox2 is knocked out in combination to them.

In order to elucidate these possible interactions between these GA3ox copies during temperature-sensitive seed germination, mRNA expression experiments could be conducted to examine the relative gene expressions of each GA3ox copies at different temperature conditions and in different mutant genotype seeds. For example, to see if GA3ox3 is in fact up-regulated more when GA3ox1 is knocked out, its expression could be quantified in GA3ox1 knock-out seeds *(ga3ox1-*) and compared to its expression in background ecotype seeds (Col). Moreover, in order to test if certain upstream PHY copies regulate specific downstream GA3ox copies, the expression of various GA3ox

genes could be tested in various PHY knock-outs and compared to their expression in the back-ground ecotype.

Ultimately the functional diversifications of these duplicated PHY and GA3ox during a single physiological process suggests that they all combine to regulate germination in response to combinations of temperature conditions. This suggests that gene duplication may be an important genetic mechanism by which seeds evolve divergent sensitivities to environmental cues. In turn, this divergence allows plants to alter the timing of its developmental transitions, such as germination, in response to complex seasonal environments. Thus, gene duplication appears to be an important genetic mechanism by which plants may achieve developmental plasticity in response to complex environmental cues and adapt in heterogeneous landscapes.

2.1 Introduction

Seed germination is just one example of a developmental transition in plants that is tightly regulated by complex combinations of environment cues. Understanding the genetic basis for this regulation is important since the timing of the developmental transitions can have significant consequences on the phenotypic expression of later life history traits and can therefore be an accurate determinant of overall lifetime fitness in a given environment (Sultan 2000; Donohue 2002) Ultimately, plants must be able to sense and respond to these combinations of environmental cues so that they can time

these developmental transitions to certain sets of environmental conditions appropriate for subsequent survival.

In the case of germination, a seed's response to environmental cues that predict seasonal environments (such as the dormancy-breaking/inducing temperature conditions used in the first part of this thesis) are particularly important for influencing subsequent life history characteristics (Donohue 2002). However, developmental responses to seasonal conditions, in general, are important for determining a plant's phenology given that they effectively govern the seasonal environments to which later life stages are exposed (Donohue 2005; Chiang, Barua et al. 2009). For example, winter-annual species germinate in response to a period of warm after-ripening followed by exposure to cold resulting in overwintering as a rosette and then flowering in the spring. (Baskin and Baskin 1972; Baskin and BASKIN 1986). Seeds that experience a prolonged exposure to cold on the other hand (i.e. – during winter conditions) may germinate, flower and disperse their seeds all in single spring season (Baskin and Baskin 1998). Ultimately, the environmental conditions a plant experiences during one developmental stage has significant consequences on the conditions experienced in life history stages. As a result, the evolution of precise responses to the environment during these developmental transitions can select for a specific phenology and contribute to plastic adaption in variable environments (Donohue 2002; West-Eberhard 2003). But what are the genetic mechanisms by which plants achieve this developmental plasticity in response to complex seasonal conditions?

Gene duplication is a common feature in plant genomes and may allow may allow plants to regulate a single physiological process in response to seasonal

environmental conditions (Hughes 1994; Lawton-Rauh 2003; Irish and Litt 2005; Flagel and Wendel 2009). The duplication of genes may arise by various independent mechanisms (that are beyond the scope of this paper) but include, for example, unequal crossing over, retroposition, or whole-genome duplications (ie – polyploidy) particularly common in the plant kingdom (Hughes 1994; Moore and Purugganan 2003; Hurles 2004; Adams and Wendel 2005). The important evolutionary consequence of gene duplication, relevant to this project however, is that duplication may create either functional redundant, functionally diversified, or even nonfunctional gene copies involved in a single physiological process (Hughes 1994; Lynch and Conery 2000; Zhang 2003).

In particular, functionally diversified gene copies, acquired through beneficial mutants following a gene duplication event, are important for plant adaption and survival in heterogeneous landscapes as they can regulate a single physiological process under various sets of environmental conditions (Zhang 2003; Irish and Litt 2005; Flagel and Wendel 2009). For example, the functional diversification of phytochromes and gibberellin3-oxidases considered earlier are just two examples of duplicated gene families involved in regulating an environmentally sensitive developmental process (in this case, temperature-dependent seed germination.) But how exactly do these pathways involving functionally diversified duplicated genes achieve developmental plasticity in variable environments and regulate a single, environmentally-sensitive physiological process?

One way these duplicated genes may regulate an environmentally-dependent process is through the diversification in their gene copies expression and/or protein

activity in response to an environmental factor (Hughes 1994; Blanc and Wolfe 2004; Irish and Litt 2005). For example, one phytochrome copy may regulate seed germination in response to cold conditions while another regulates its response to warm conditions. Conversely, a single gene copy may be functionally diversified in its sensitivity to two environmental factors. For example, if a GA3ox's gene expression is regulated by a certain imbibition (dark) temperature while its protein activity is regulated by certain germination (light) temperture. But how do *combinations* of duplicated genes (such as PHY and GA3ox) with varying functional diversifications regulate a single physiological process (ie – germination) in response to combinations of environmental factors? Moreover, how can these pathways with upstream and downstream duplicated genes restrict a physiological process so that it occurs in specific sets of environmental conditions appropriate for subsequent survival?

To answer these questions, a basic two-step genetic pathway model was constructed in which a single upstream gene regulates one downstream gene and that downstream gene regulates a single physiological response to the combination of two environmental variables[2]. In the model, both upstream and downstream genes have two duplicated copies, which can be independently regulated (to varying degrees) by one or both of these two environmental factors. Thus, this model allows us (in a very basic sense) to investigate how a physiological response is restricted to certain combinations of environmental factors depending on whether the upstream versus downstream duplicated genes controlling it are environmentally sensitive and/or functionally

[2] Dr. Kathleen Donohue was responsible for creating the following model's parameters and equations. I was responsible for coming up with comparisons to test the effect of environmental sensitivity/diversification within these equations on the model output and analyzing these results within the context of gene duplication and plant development. We also worked together in creating the model's basic overall pathway structure (e.g. – 'independent' versus 'pooled' regulation of the downstream gene).

divergent in their environmental sensitivities. The model also takes into consideration two possible pathway structures in which a duplicated upstream gene can regulate a duplicated downstream gene. In the 'independent regulation' of the downstream gene, the two upstream copies each regulate a single downstream copy, whereas in the 'pooled regulation', the upstream copies activity combine (or pool) to effect each downstream copy's activities.

Ultimately, this model is relevant to the experimental assays discussed in the first part of this thesis as it can be used to consider phytochromes and gibberelin3-oxidases' functionally diversified roles during temperature-dependent seed germination. In the case of germination, upstream PHY copies are known to regulate downstream GA3ox copies while both PHY and GA3ox genes are independently regulated by stratification temperature (environmental variable 1) and germination temperature (environmental variable 2). The physiological response (i.e. -seed germination) is then is a function of the total 'output' of the downstream genes (i.e.- combined GA3ox levels). The real purpose of this model, however, is to explore how duplicated upstream versus downstream genes, in general, can regulate an environmentally-dependent physiological process depending on their own environmental sensitivities and possibly diversified gene copies.

2.2 Modeling Equations & Methods

The purpose of this model is to investigate how a physiological process may be restricted in response to combinations of two environmental factors depending on the duplication and/or environmental sensitivities of the upstream and downstream genes

regulating it. Each upstream and downstream gene has two duplicated copies which can be independently regulated by two environmental factors. The upstream gene's activity is only a function of the two environmental factors while the down stream gene's activity is a function of both the environment *and* the upstream gene's activity. The final physiological outcome is then a function of the downstream gene's activity. Finally the model is structured so that we can compare the effects on the physiological outcome when each downstream gene copy is regulated independently by a single upstream copy versus when each downstream gene copy is regulated by the combined (or 'pooled') activities of both upstream copies. Schematic depictions of these pathways are shown in Fig. 8 and 9.

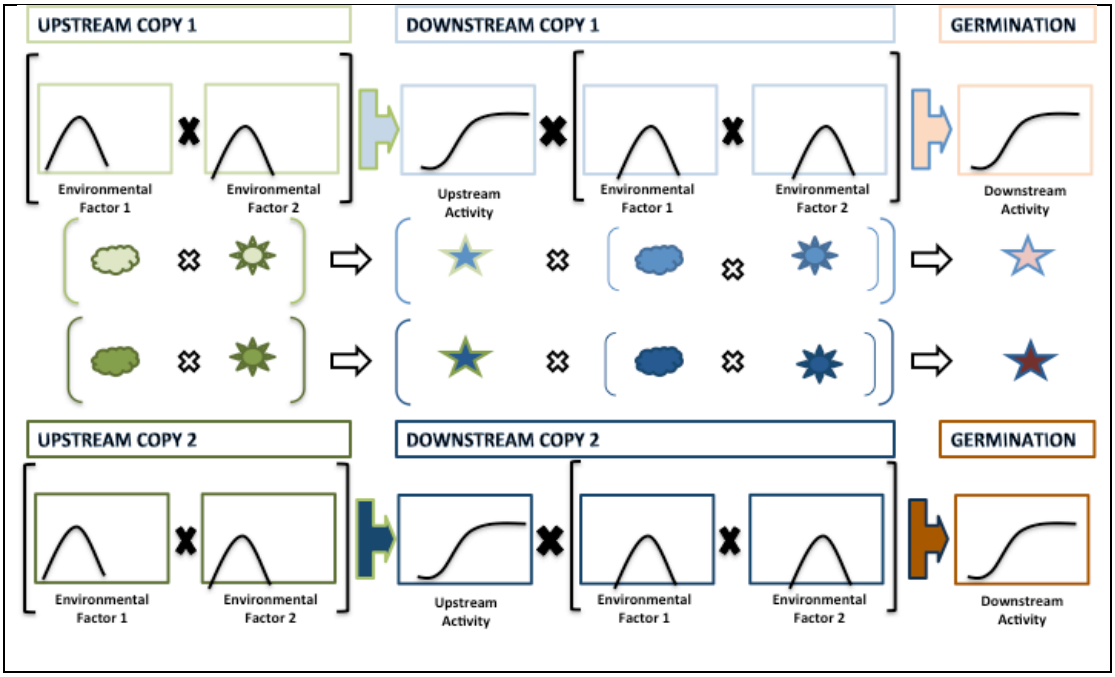

Fig. 8 Depiction of *independent regulation* of each downstream gene copy by a single upstream gene copy.

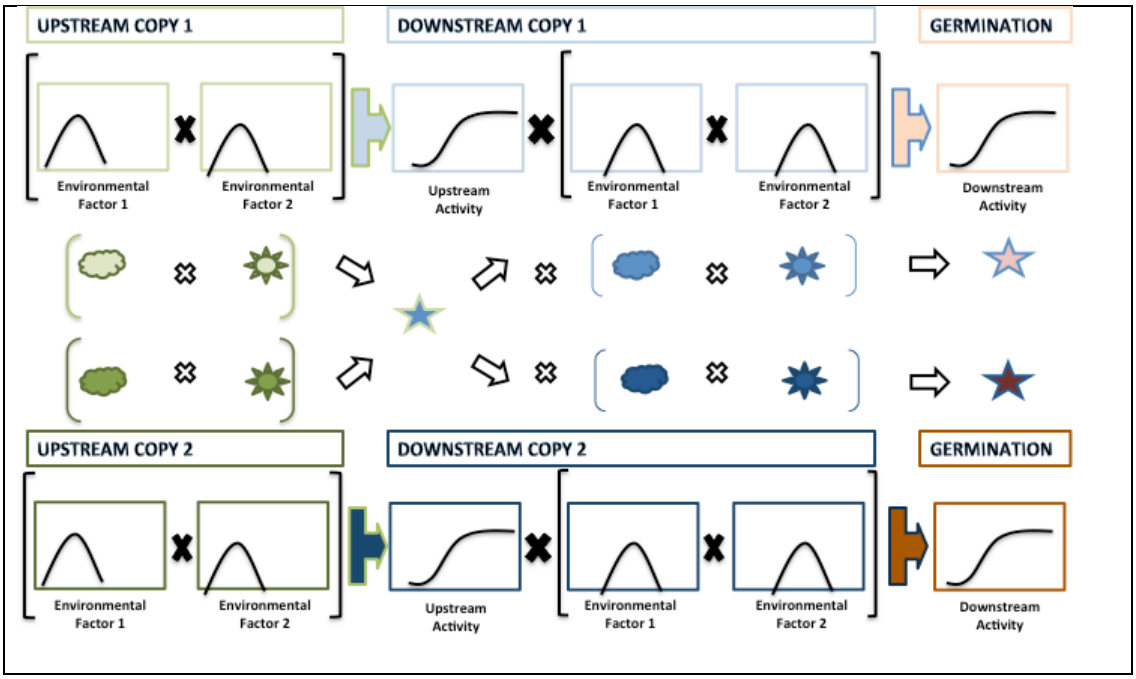

Fig. 9 Depiction of *pooled regulation* of downstream gene copies by a duplicated upstream gene. The total activity of each upstream gene copy combines to affect each downstream copy equally, as illustrated by the single blue star in Fig. 9.

Here, the model was used to address the following questions:

1) *How does environmental sensitivity in non-divergent upstream versus downstream gene copies restrict a physiological response to combinations of environmental factors?* In terms of the model, 'non-divergent' genes represent duplicated genes with identical sensitivities (or lack-thereof) to each environmental factor[3]. This question was tested by comparing the physiological responses to the environment when: a) only the upstream gene is environmentally sensitive to both environmental factors b) when only the downstream gene is sensitive to both environmental factors c) when both the upstream and downstream genes are sensitive to a single environmental factor and d) when both the upstream and downstream genes are sensitive to both environmental factors.

[3] This generates the same model output as if there were only one upstream and one downstream gene copy. Thus, testing non-divergent copies is essentially testing *non-duplicated* genes as well

2) *How does identical versus divergent environmental sensitivities in duplicated upstream and/or downstream genes restrict a physiological response to combinations of environmental factors?* This was tested by comparing the model output responses to the environment when: a) duplicated upstream and downstream genes have diversified sensitivities to a single environmental factor, b) only the duplicated upstream has diversified sensitivities to both environmental factors c) only duplicated upstream gene has identical sensitivities to both environmental factors and d) both the duplicated upstream and downstream genes have diversified sensitivities to both environmental factors.
3) *How does the 'independent' versus 'pooled' regulation of the downstream gene restrict a physiological response to combinations of environmental factors when there is gene duplication and/or divergent sensitivities in the upstream versus downstream genes?* This was tested by comparing the same four pathways described in question 2 except with both downstream gene copies being regulated by the combined activities of both upstream gene copies.

To answer these questions, comparisons one and two were conducted assuming the 'independent' regulation of each downstream copy by a single upstream copy, while question three was tested assuming the 'pooled' regulation of each downstream copy by the combined upstream copy activities. Note, that this model also assumes that a single gene can be influenced by two environmental factors. This can occur when more than one environmentally sensitive cis-regulatory region regulates a gene, for example, or when one environmental variable regulates a gene's expression and the other

environmental variable regulates that gene's RNA stability or protein activity. For example, in the case of seed germination, stratification temperature might regulate a gene's expression while germination temperature might regulate that gene's protein activity. In this model, each environmental variable, e_1 and e_2 independently regulate the upstream gene's output (A_u) and/or the downstream gene's output (A_d).

Upstream Gene

The upstream activity is regulated by two environmental factors given by the following parabolic functions in Eqns. 1 and 2. The first describes the gene output when environmental conditions are below the optimum, and the second describes gene output when conditions are above the optimum. The output of the upstream gene, contingent on environmental factor 1, is:

$$A_u(e^1) = \frac{1}{\alpha_{0,u}(e1_{o,u} - e1_{a,u})^2 + 1}, \quad e^l{}_{a,u} < e^l{}_{o,u} \qquad \text{[Eqn. 1.1]}$$

$$A_u(e^1) = \frac{1}{\alpha_{1,u}(e1_{o,u} - e2_{a,u})^2 + 1}, \quad e^l{}_{a,u} > e^l{}_{o,u} \qquad \text{[Eqn. 1.2]}$$

The first subscript indicates whether the environmental value is the optimum (o) or actual (a) value, and the second subscript indicates whether the gene is upstream (u) or downstream (d). Therefore, $e^l{}_{o,u}$ is the upstream gene's optimum environmental 1 value and $e^l{}_{a,u}$ is the actual environmental value. When the actual value is below the optimum, the upstream gene's activity approaches zero at a rate denoted by $\alpha_{0,u}$ (Eqn. 1.1). When the actual value is above the optimum, the upstream gene's activity controlled by environmental factor 1 approaches zero at a rate denoted by $\alpha_{1,u}$ (Eqn. 1.2).

Similarly, the output of the upstream gene, contingent on environmental factor 2, is:

$$A_u(e^2) = \frac{1}{\beta_{0,u}(e2_{o,u} - e2_{a,u})^2 + 1}, \; e^2{}_{a,u} < e^2{}_{o,u} \qquad \text{[Eqn 2.1]}$$

$$A_u(e^2) = \frac{1}{\beta_{1,u}(e^2{}_{o,u} - e^2{}_{a,u})^2 + 1}, \; e^2{}_{a,u} > e^2{}_{o,u} \qquad \text{[Eqn. 2.2]}$$

where $e^2{}_{o,u}$ is the upstream gene's optimal environmental 2 value and $e^2{}_{a,u}$ is the actual environmental 2 value. Like the ' α' values from Eqn 1, $\beta_{o,u}$ and $\beta_{1,u}$ are the rates at which the upstream gene activity controlled by environmental factor 2 ($A_u(e_2)$), approaches zero when it's the actual environmental 2 value is below and above the optimum, respectively.

As one can see by these equations, the sensitivity to both environmental variables has optimal values, $e^1{}_o$ and $e^2{}_o$, which approach zero at a rate denoted by α and β respectively. This rate of decline above and below the optimum can be symmetrical (when α_0 and α_1 or β_o and β_1 are equal) or unsymmetrical (when α_0 and α_1 or β_o and β_1 are not equal).

The upstream gene's total activity, A_u, is simply the gene activity controlled by environmental factor 1 multiplied by the gene activity controlled by environmental factor 2 (eqn 3).

$$A_u = A_u(e^1) \times A_u(e^2) \qquad \text{[Eqn. 3]}$$

Downstream Gene

The level of downstream gene expression (E_d) produced by the upstream gene's activity (A_u) is given by the following sigmoid function (eqn 4).

$$E_d(A_u) = e^{-e^{(\delta_0-(\delta_1*A_u))}} \quad \text{[Eqn. 4]}$$

where δ_0 determines the steepness of the sigmoidal curve and δ_1 determines the point at which the curve begins to increase.

The downstream gene's total activity, A_d, (eqn 7) is then the combined gene activity that is a function of environmental variables 1 and 2 (eqns 5 and 6) multiplied by the downstream expression that is a function of upstream activity (eqn 4). The downstream gene's activity, contingent on environmental factor 2, is then:

$$A_d(e^1) = \frac{1}{\alpha_{0,d}(e^1{}_{o,d}-e^1{}_{a,d})^2+1}, \quad e^1{}_{a,d} < e^1{}_{o,d} \quad \text{[Eqn. 5.1]}$$

$$A_d(e^1) = \frac{1}{\alpha_{1,d}(e^1{}_{o,d}-e^1{}_{a,d})^2+1}, \quad e^1{}_{a,d} > e^1{}_{o,d} \quad \text{[Eqn. 5.2]}$$

where $e^1{}_{o,d}$ is the downstream gene's optimal environmental 1 value and $e^1{}_{a,d}$ is the actual environmental 1 value . Like the upstream gene's activity from Eqn. 1, $\alpha_{0,d}$ and $\alpha_{1,d}$ are the rates at which downstream gene's activity due to environmental factor 1, $A_d(e^1)$, approaches zero when that environmental 1 value is below and above the optimum, respectively.

Finally, the downstream gene's activity contingent on environmental factor 2 is :

$$A_d(e^2) = \frac{1}{\beta_{0,d}(e^2{}_{o,d} - e^2{}_{a,d})^2 + 1}, \; e^2{}_{a,d} < e^2{}_{o,d} \qquad \text{[Eqn. 6.1]}$$

$$A_d(e^2) = \frac{1}{\beta_{1,d}(e^2{}_{o,d} - e^2{}_{a,d})^2 + 1}, \; e^2{}_{a,d} > e^2{}_{o,d} \qquad \text{[Eqn. 6.2]}$$

where $e^1{}_{o,d}$ is the downstream gene's optimal environmental 2 value, $e^1{}_{a,d}$ is the downstream gene's actual environmental 2 value and β_o and β_1 are the rates at which the downstream gene's activity approaches zero when the actual environmental 2 value is below and above the optimum, respectively.

The total downstream activity (A_d) is equal to the downstream expression controlled by upstream activity, $E_d(A_u)$, multiplied by the combined downstream gene activities controlled by environmental variables 1 and 2 (Eqn 7).

$$A_d = E_d(A_u) \times A_d(e^1) \times A_d(e^2) \qquad \text{[Eqn. 7]}$$

Physiological Outcome

The physiological outcome (P) is a function of the total downstream activity (Eqn 8).

$$P(A_d) = e^{-e^{(\gamma_0 - (\gamma_1 * L_{1,2}))}} \qquad \text{[Eqn. 8]}$$

The following model outputs give this physiological outcome value as a function of the two environmental factor's values (ie – in a two-dimensional surface chart with one environmental factor on the x-axis and the other on the y-axis). In the model, each environmental factor is scaled from 1 to 20, and the modeling parameters are chosen so

that the physiological outcome values are between 0 and 1 (to represent the proportion of a population exhibiting a given physiological response within a certain combination of the two environmental factors' values).

2.3 Model Results

2.3.1 Environmental Sensitivity with No Diversification of Gene Copies

The following four comparisons test the effect of environmental sensitivity in non-divergent (ie- non-duplicated) upstream versus downstream genes regulating an environmentally dependent physiological response (Fig. 10). In each of these analyses, the variables delta (δ) and gamma (γ), which determine the downstream expression as a function of upstream activity and the physiological response (eg – germination proportion) as a function of downstream activity, respectively, are held constant.[4]

The first scenario (1A) shows the physiological outcome as a function of two environment factors when only upstream copies are environmentally sensitive (ie – neither downstream copies are environmentally sensitive and both upstream gene copies have identical sensitivities to both environmental factors). The second scenario (1B) shows results with the exact same environmental sensitivities as 1A, but only the downstream copies are sensitive. The third scenario (1C) also tests the same environmental sensitivities, but the upstream copies are identically sensitive to one environmental factor while the downstream copies are identically sensitive to the other environmental factor. Finally, the fourth scenario (1D) shows results with the same

[4] NOTE: Delta and gamma values were chosen so physiological response values would be between 0 and 1 (to reflect germination proportion).

environmentally sensitivity as A through C in both the upstream and downstream copies (Fig.10).

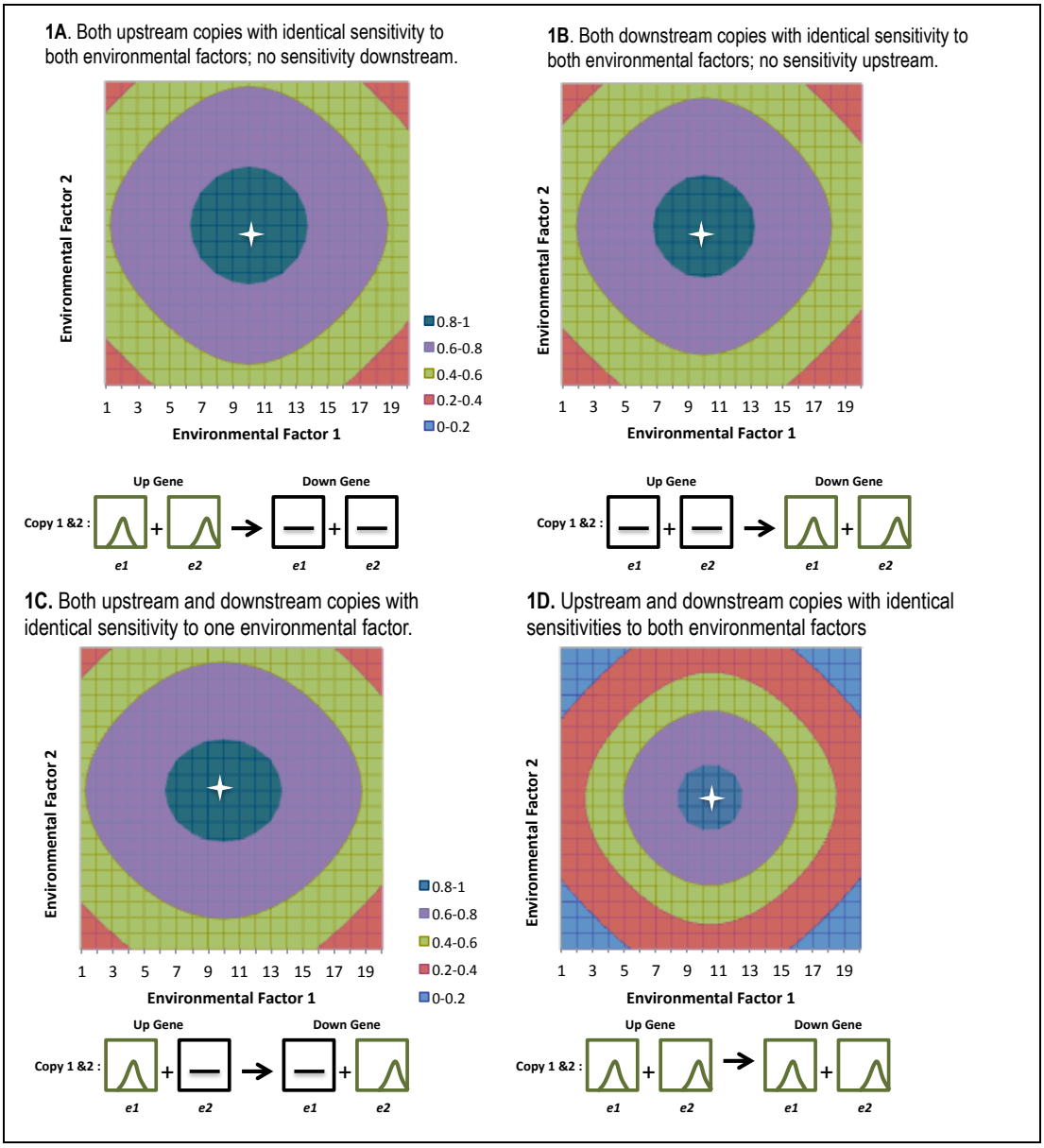

Fig. 10 Effect of environmental sensitivity in upstream vs. downstream gene copies when there is no gene duplication (or both gene copies have identical sensitivities as shown in the icons above which show each gene copy's activity as a function of 2 environmental factors. White star in each panel represents the optimal environmental factors (10 and 12 for e_1 and e_2, respectively). Model parameters: $\alpha_0 / \alpha_1 = 0.01$; $B_0 / \beta_1 = 0.01$; δ_0 , $\delta_1 = 1, 2$; γ_0 , $\gamma_1 = 1,2$.

Without gene duplication (or when duplicated gene copies have not diverged in their sensitivities to either environmental factor as shown here) there is little effect on the

restriction of the physiological outcome around optimal conditions when only the upstream versus only the downstream gene is environmentally sensitive. Similarly, there is little difference when non-divergent upstream and downstream genes are each sensitive to a different environmental factor (1C) versus the upstream or downstream gene is sensitive to both (1A /1B). Nevertheless, environmentally sensitivity downstream does result in a slightly "tighter" restriction around the optimal conditions (1B vs. 1A). Moreover, When both the upstream and downstream copies have identical environmental sensitivities, there is a much more significant restriction of the physiological outcome's response around the optimal environmental conditions (Fig. 10, 1D).

In the Fig. 10 comparisons, however, the delta values (which determine the effect of upstream activity on downstream expression) were set equal to the gamma values (which determine the effect of downstream activity on the physiological outcome responses to environmental conditions). In other words, the upstream gene's "downstream effect" on the downstream gene expression (delta) is greater than the downstream gene's 'downstream effect' on the physiological response (gamma). Thus, to illustrate more clearly the possible differences upstream versus downstream sensitivities can have on the physiological outcome, the delta values were increased to 5 and 7 and the same comparisons as described for Fig. 10 were performed in the model (Fig. 11).

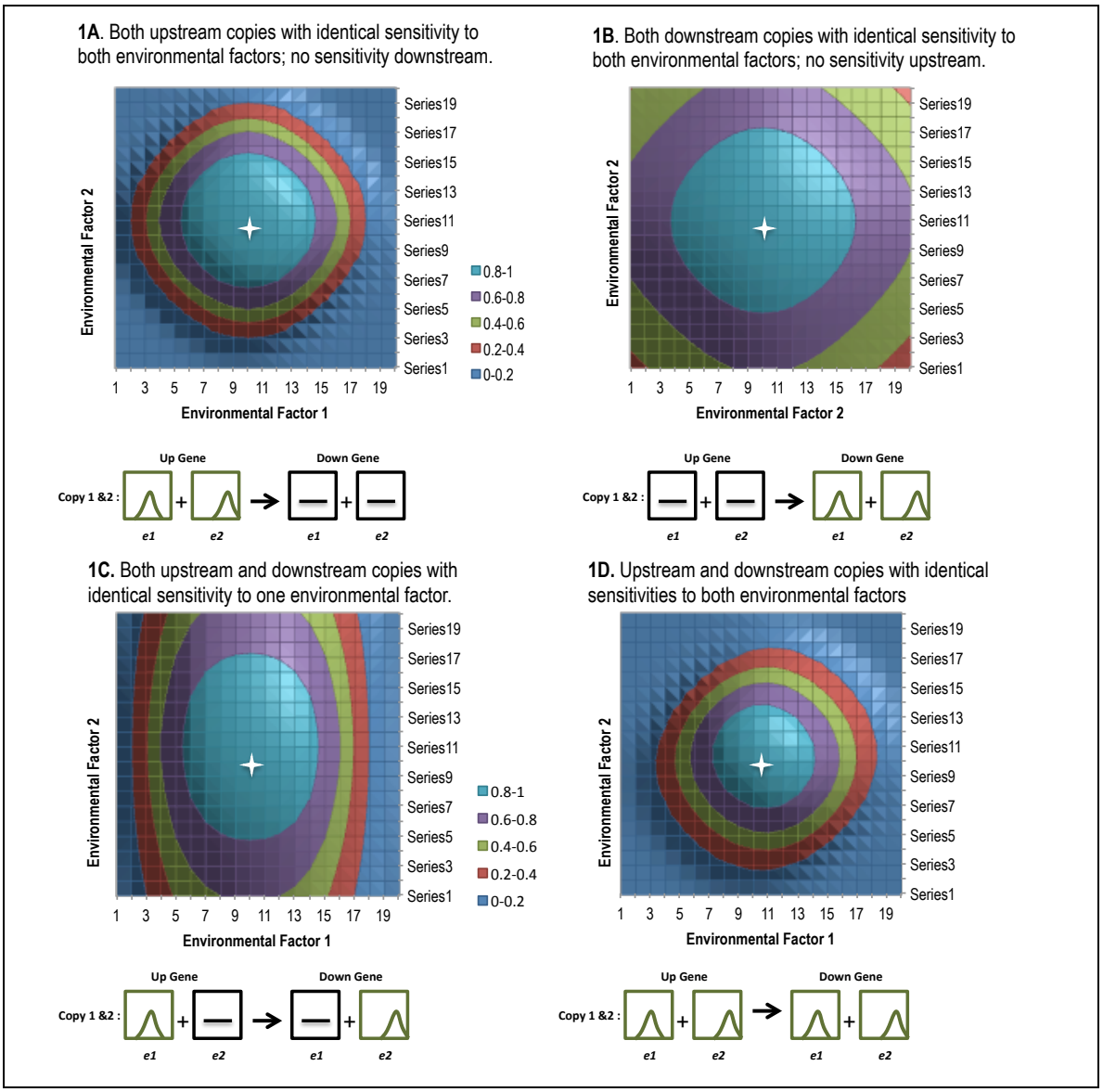

Fig. 11 Pathways 1A through 1D with the same modeling parameters as in fig except with the delta values (5,7) set higher than the gamma values (1,2).

Unsurprisingly, in these outputs, environmental sensitivity in the upstream gene (1A) has a greater effect in restricting the physiological response around the optimal environmental conditions than downstream sensitivity (1B). In other words, when only the upstream gene is environmentally sensitive (1A), physiological responses decrease at a slightly faster rate away from the optimal environmental conditions specified in the model (e_1= 10, e_2 = 12) than they do when only the downstream gene is sensitive (1B).

This suggests that when there is no diversification with a gene, different responses to the environment when there is upstream versus downstream sensitivity, is contingent on whether the upstream versus downstream gene has a greater 'downstream effect.' When the upstream gene's downstream effect is greater (delta > gamma) upstream sensitivity (1A) restricts responses to the environment more precisely than downstream sensitivity (1B) sensitivity. This point is proven again in 1C where the physiological response is more tightly restricted by environmental factor 1 (to which the upstream gene is sensitive) versus environmental factor 2, to which the downstream gene is sensitive. Thus, differences in restriction to one environmental factor over the other can occur if delta ≠ gamma.

Nevertheless, when the delta values are set higher than the gamma values (ie- the upstream activity has a greater effect on downstream expression, than downstream activity has on the physiological outcome) identical sensitivities in both the upstream and downstream genes still results in the greatest overall restriction of response to the environment (Fig 11, 1D) as seen in Fig. 10. Thus, both of these comparisons show that when there is no diversification among gene copies, identical sensitivities in both the upstream and downstream genes results in the greatest restriction of a physiological response around the combination of optimal environmental values.

2.3.2 Environmental Sensitivity with Diversification of Gene Copies

The next four comparisons illustrate how divergence in the environmental sensitivities of duplicated genes affects environment-dependent physiological outcomes. Scenario 2A tests the effect on germination proportions when both upstream and

downstream genes have evolutionarily diverged, as a result of gene duplication, in their sensitivity to a single environmental factor. Here, the upstream copies have diverged in their sensitivity to environmental factor 1while the downstream copies have diverged in their sensitivity to environmental factor 2. All four gene copies are environmentally sensitivity however. Scenario 2B tests the effect on the physiological outcome when the upstream gene has diverged in its sensitivity to both environmental factors, while the downstream gene, copies have identical environmental sensitivities (ie – both downstream copies have identical environmental sensitivities). Scenario 2C tests the effect on the physiological outcome when the duplicated downstream gene has divergent sensitivities to both environmental factors and the upstream gene copies have identical environmental sensitivities. Finally the 2D scenario examines the effect on the physiological outcome when both upstream and downstream genes have diverged with identical sensitivities to both environmental factors (Fig. 11). In all of these scenarios, each downstream gene copy is regulated independently by a single upstream gene copy.

With the delta values greater to the gamma values (i.e. – upstream activity has stronger effect on downstream expression than downstream activity has on the physiological response downstream activity) we find that upstream diversification (1B, Fig. 12) has a more pronounced effect (in terms of restricting the physiological response around the optimal environmental conditions) than downstream diversification (1C, Fig. 12).

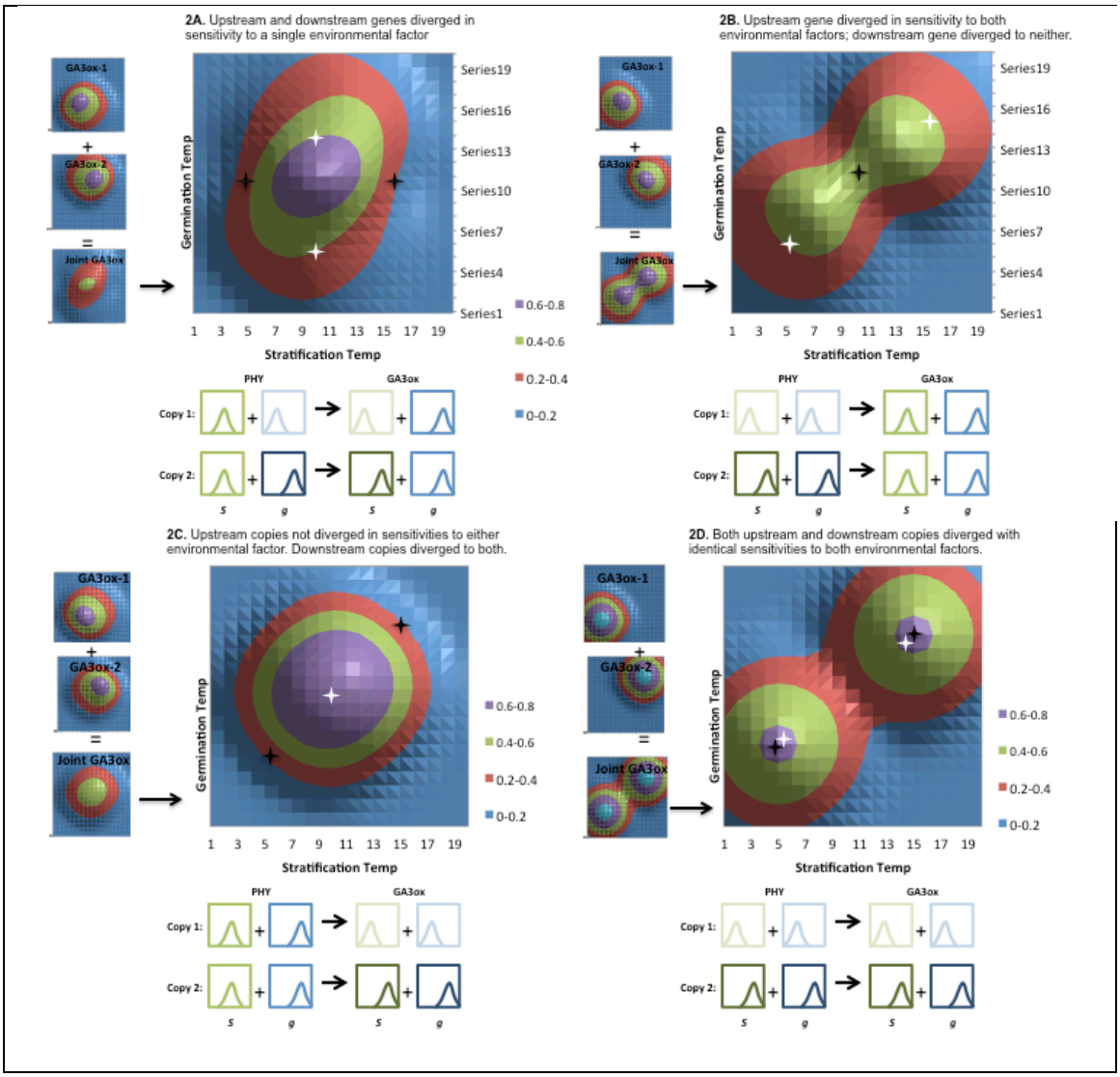

Fig. 12 Pathway comparisons to test effect of environmental sensitivity with gene duplication on germination in upstream vs. downstream gene copies. The side panels show the individual and combined GA3ox concentrations that determine the final germination outcome. White and black stars show environmental optimums for upstream and downstream copies respectively. (Model parameters used in each of the four comparisons: α_0 / α_1= 0.01 ; B_0 / β_1 = 0.01; δ_0 , δ_1= 5, 7; γ_0 , γ_1= 1,2; non-divergent e_1 = 10; non-divergent e_2= 12; divergent e_1= 5,15; e_2 = 8,16).

The highest possible physiological response over the largest range of environmental conditions occurs when only the downstream gene has divergent sensitivity (2C). This can be explained by examining the individual downstream copy's expressions (e.g. – levels of GA3ox shown in side panels) that result as a function of a divergent (2B) or non-divergent (2C) upstream gene (Fig. 12). When there is no

diversification in the upstream gene's environmental sensitivity (2C), both downstream copies expression as a function of upstream gene activity are the same. Thus, the only factor contributing the restriction of the physiological response to the environment is the divergence in downstream sensitivity. As we saw in Fig. 11 has much less of an effect on the physiological outcome than upstream gene's sensitivity when delta > gamma.

Nevertheless, downstream divergence still contributes to the restriction of physiological responses to combinations of environmental factors, particularly when the downstream gene diversification to both environmental factors is identical to the upstream diversification. (Fig. 13. 2D). Once again therefore, we can conclude that the greatest restriction of a physiological response around its optimal environmental conditons results when both upstream and downstream genes have identical sensitivities to both environmental factors (regardless of whether each gene is diversified (ie – duplicated) or not). However, when the copies of a both genes do have diversified sensitivities, identical physiological responses (ie – multiple peaks) may be observed to multiple combinations of environmental factors (1D).

2.3.3 Independent vs. Pooled Regulated of Downstream Copies

To investigate the effects of independent versus pooled regulation of the downstream gene by the upstream copies, I will refer back to the pathways described in section 2.2.2 (all of which had 'independent' regulation of the downstream gene). 'Pooled' regulation of the downstream gene by the upstream gene only has an effect on final physiological response only when the upstream copies have diverged in their

sensitivities to one or both of the environmental factors. For example, pooling the upstream activities in pathway 2C from the previous analysis does not change the final physiological outcome in response to the environmental factors. When the upstream copies are pooled in 2D however, physiological responses are lowered and spread out between the two sets of optimal environmental conditions (ie – $e_{1,u}$=5, $e_{2,u}$ =8 & $e_{1,d}$=10, $e_{2,d}$=16).

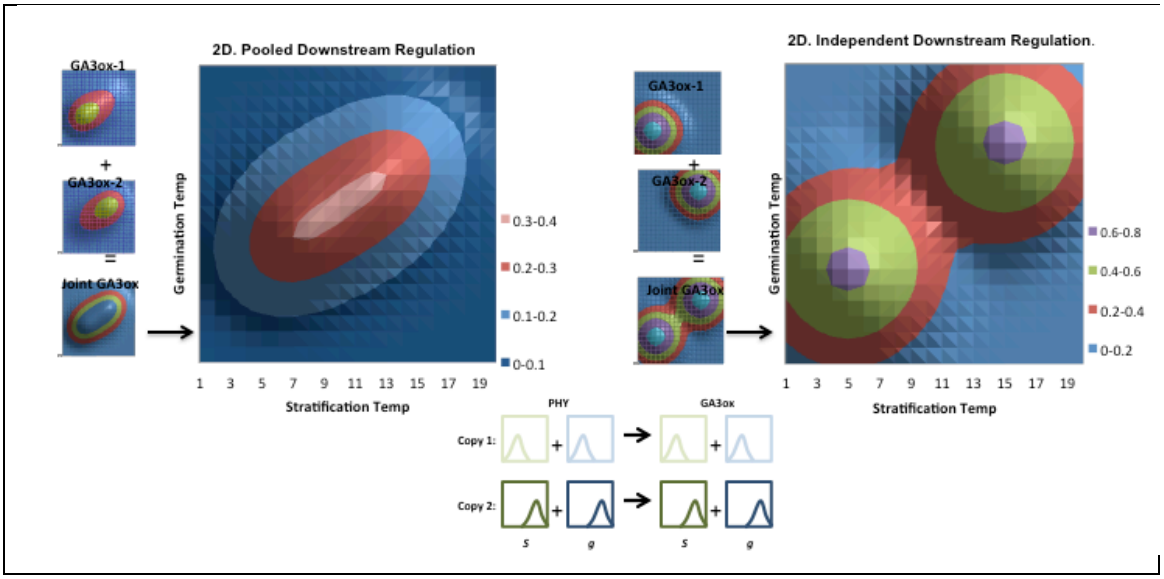

Fig. 13 Pathway 2D final germination outcomes when downstream GA3ox copies are either regulated by both upstream PHY copies "pooled" activity or independently regulated by only one upstream copy. (Model parameters same as 2D pathway in Fig. 12)

In the independent regulation of the downstream gene, upstream copy 1 regulates downstream copy 1 and upstream copy 2 regulates downstream copy 2. As a result, the difference between 'pooled' vs. 'independent' regulation of the downstream gene is even more significant depending on whether the same copy of the upstream and downstream genes have identical sensitivities or not . In the scenario 2D for example, upstream copy 1 and downstream copy 1 have identical sensitivities, while upstream and downstream copy 2 have identical sensitivities. Thus environmental sensitivities are diversified within a gene, but these diversifications are identical between genes. If the

environmental sensitivities of downstream copy 1 and 2 are switched however in scenario 2D however (while keeping therest of the model parameters the same) the physiological responses are greatly reduced and no longer confined to two distinct sets of environmental conditions (call this pathway 2E; Fig. 14). There is no difference between scenarios 2D and 2E if the downstream gene's are regulated by the combined (pooled) activities of the upstream gene. Therefore, pooled regulation may result in more precise restriction of physiological responses around the optimal environmental conditions, but only when the duplicated upstream and downstream gene copies are *not* identically diversified to the two environmental factors (as in scenario 2E, Fig. 14).

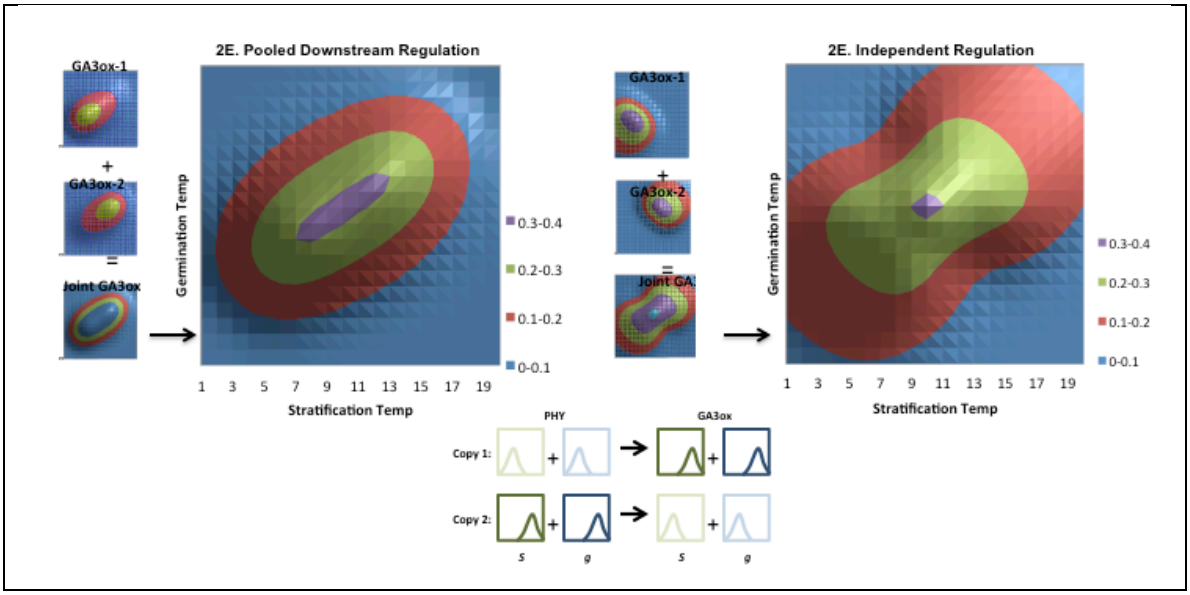

Fig. 14 Germination outcome of 2E pathway when downstream copies are subject to pooled vs. independent regulation by the upstream copies. Same modeling parameters as 2D pathway except sensitivities of downstream copies are reversed (see iconic depiction below main panels).

2.4 Discussion

When there is no diversification of the upstream or downstream copies with respect to their environmental sensitivities (scenarios 1A – 1D), the most precise restriction of the physiological response around optimal environmental conditions environmental factors is achieved when both the upstream and downstream copies have identical sensitivities to both environmental factors (Fig. 10/11, 1D). This is true regardless of whether the upstream gene has a greater (delta > gamma) or equal (delta = gamma) 'downstream effect' than the downstream gene.

When the upstream gene's 'downstream effect' is larger however (Fig. 11) there is a *less precise* restriction of response to optimal conditions regardless of whether the upstream versus downstream genes are sensitive (1A – 1D Fig. 10 vs. Fig. 11) In other words, there are fewer combinations of environmental factors that result in the highest possible physiological response when the upstream gene has a stronger "downstream effect" than the downstream gene (delta > gamma, Fig. 11). However only when the upstream and downstream gene's 'downstream effects' are not equal (delta ≠ gamma) are significant differences in responses to upstream versus downstream sensitivities observed in the model outputs (Fig. 11 1A vs. 1B vs 1C). Conversely ,when each gene has identical 'downstream effects' (delta =gamma, Fig. 10) sensitivity in the upstream versus downstream (1A vs. 1B vs. 1C) does not have a substantial effect on the physiological response to the environment.

Thus, if a physiological outcome needs to be as precisely restricted to a combination of environmental factors as possible *without* the diversification of gene copies, identical 'downstream effects' of the upstream and downstream genes may be

selected for by the environment (Fig. 10 1D > Fig. 11 1D). If however, a plant needs to restrict its physiological responses to one environmental factor over the other, differences in the 'downstream effects' of the two genes may be selected for. This could result in the evolution of a pathway such as 1C (Fig. 11) where the upstream's sensitivity to environmental factor 1 has a stronger effect on restricting the physiological outcome than the downstream gene's sensitivity to environmental factor 2.

This "restriction" of physiological responses (e.g. – seed germination) around optimal environmental conditions is important in an ecological and evolutionary context. Assuming germination, for example, under optimal conditions is a key determinant of that plant's later survival and fitness in an environment, selection may favor a pathway with identical upstream sensitivity and downstream sensitivities (regardless of diversification) since fewer seeds would germinate under "non-optimal" conditions possibly compromising subsequent survival. Ultimately, seeds must restrict germination (and other developmental processes) to the widest possible set (or sets) of environments conditions appropriate for their survival, without being overly restrictive. Not enough restriction and plants may develop in environmental conditions that aren't well suited for subsequent survival or that don't maximize their overall lifetime fitness. Too much restriction could be just as costly however. For example, if their "window of opportunity" to develop is too narrow, then plants may be unable to develop at all or never get the chance to adapt in response to shifting environmental conditions (resulting from climate change, for example).

Ultimately, the ecological environment in which a plant is exposed determines how a plant should restrict a given developmental transition in response to the

combinations of environmental cues. Thus, a plant's environment may also determine where environmentally sensitivity and/or diversification evolves in duplicated gene families responsible for regulating these transitions. To this extent, if we know a plant's environment we can predict what sort of basic pathways involving duplicated genes might evolve in order to regulate a given physiological response to combinations of environmental factors. For example, if the environment is very stable and climate conditions aren't likely to fluctuate much from year to year, identical sensitivities to multiple environmental factors in upstream and downstream duplicated gene families might be selected for. This would result in the most precise restriction of a response to its optimal conditions regardless of whether upstream or downstream activities have a greater "downstream consequence." (Fig. 10 & 11, 1D scenario). On the other hand, if the environment is more variable and its conditions more unpredictable, a plant might evolve different sensitivities to multiple environmental factors in upstream and downstream genes (Fig. 11, 1C).

This difference in identical versus divergent sensitivities is even more pronounced when there is diversification within upstream and downstream gene copies (1D vs. 2D). Moreover, the model predicts that when there are duplicated genes with environmentally diversified copies, multiple physiological response "peaks" can arise in to multiple sets of optimal environmental conditions (as seen by the two "optimal peaks" seen in scenario 2D's output, Fig. 12). The evolution of multiple combinations of optimal environmental factors is of particular ecological importance when considering how plants might adapt in variable environments through developmental plasticity. Regulating developmental transitions (such as the timing of seed germination) in

response to different environmental conditions allows plants to select for a given phenology that is most well suited to a particular environment. In order to achieve this, duplicated genes in the germination pathway (such as PHY and GA3ox) may evolve functionally diversified role in response to different combinations of environmental cues (for example temperatures in the dark (imbibition) and in the light) that accurately reflect a given season. Thus duplicated gene families, particularly those with environmentally diversified roles, may be an important 'genetic prerequisite' for plants of a single genotype to alter the timing of its developmental transitions (i.e. – achieve developmental plasticity) in response to complex sets of environmental cues and adapt 'plastically' in heterogeneous landscapes.

According to this model, identical environmentally sensitivities in duplicated upstream and downstream genes results in the greatest restriction of a physiological response around its set(s) of optimal environmental factors (1A vs. 1D). This restriction is most precise when upstream and downstream gene copies have identically diversified sensitivities versus non-diversified identical sensitivities (2D vs. 1D). Moreover, diversified sensitivities in duplicated genes allows for multiple physiological "peaks" in response to multiple combinations of optimal environmental conditions. Environmentally sensitive duplicated genes that are not diversified, however, are unable to create these multiple "peaks" under optimal conditions (scenarios 1A – 1D). This suggests the importance of environmentally diversified duplicated genes for the evolution of precise developmental responses to multiple combinations of environmental conditions. Thus, gene duplication is one mechanism by which plants may survive and adapt in complex seasonal environments – achieved through the evolution of

environmentally diversified gene copies able to regulate a single developmental process in response to combinations of environmental cues.

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